Paper-based assays for urine analysis

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(Received 18 July 2017; accepted 29 September 2017; published online 17 October 2017)

A transformation of the healthcare industry is necessary and imminent: hospital-centered, reactive care will soon give way to proactive, person-centered care which focuses on individuals' well-being. However, this transition will only be made possible through scientific innovation. Next-generation technologies will be the key to developing affordable and accessible care, while also lowering the costs of healthcare. A promising solution to this challenge is low-cost continuous health monitoring; this approach allows for effective screening, analysis, and diagnosis and facilitates proactive medical intervention. Urine has great promise for being a key resource for health monitoring; unlike blood, it can be collected effortlessly on a daily basis without pain or the need for special equipment. Unfortunately, the commercial rapid urine analysis tests that exist today can only go so far—this is where the promise of microfluidic devices lies. Microfluidic devices have a proven record of being effective analytical devices, capable of controlling the flow of fluid samples, containing reaction and detection zones, and displaying results, all within a compact footprint. Moving past traditional glass- and polymer-based microfluidics, paper-based microfluidic devices possess the same diagnostic ability, with the added benefits of facile manufacturing, low-cost implementation, and disposability. Hence, we review the recent progress in the application of paper-based microfluidics to urine analysis as a solution to providing continuous health monitoring for proactive care. First, we present important considerations for point-of-care diagnostic devices. We then discuss what urine is and how paper functions as the substrate for urine analysis. Next, we cover the current commercial rapid tests that exist and thereby demonstrate where paper-based microfluidic urine analysis devices may fit into the commercial market in the future. Afterward, we discuss various fabrication techniques that have been recently developed for paper-based microfluidic devices. Transitioning from fabrication to implementation, we present some of the clinically implemented urine assays and their importance in healthcare and clinical diagnosis, with a focus on paper-based microfluidic assays. We then conclude by providing an overview of select biomarker research tailored towards urine diagnostics. This review will demonstrate the applicability of paper-based assays for urine analysis and where they may fit into the commercial healthcare market. *Published by AIP Publishing.* https://doi.org/10.1063/1.4996768

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I. INTRODUCTION

There is an unmet need for a transformation in the healthcare system from reactive and hospital-centered care to a more proactive approach, encompassing preventive, evidence-based, and person-centered care.[1] The focus must shift from disease to well-being. To support this much-needed transformation, there is an imminent demand for low-cost, compact, and transformative technologies to perform hourly, daily, or continuous health measurements across the population. Only rigorous scientific innovation can realize such next-generation technologies, which promises to improve patients' well-being, decrease the cost of care, and take on ever-present medical challenges.

In the United States alone, healthcare costs are astronomically high and continue to increase every year, exceeding $9403 per capita in 2014.[2] In 2014, the healthcare expenditure in the United States represented 17.1% of its gross domestic product—the highest healthcare expenditure among all countries in the world. This trend is expected to continue in the coming years [Fig. 1(a)].[3–5] Healthcare spending comprised 52.9% from private insurance companies and 20.7% from federal government aid. Federal aid is further divided into various programs, including Medicare for the elderly population over the age of 65, Medicaid for low-income people, and Children's Health Insurance Program for low-income families with children.[6] Additionally, a portion of the population does not have either private or government insurance, and consequentially hold off on seeking treatment until there is a dire need [Fig. 1(b)].

In a study reported by Health Affairs, it was shown that a 90% increase in specific preventative screenings back in 2006 would have saved more than 2 million lives without a significant increase in healthcare costs (in fact, a 0.2% decrease in costs was estimated).[7] In addition to the improvement in public health, healthcare cost savings could be drastically improved by further research focused on the development and implementation of low-cost preventative care methods and tools. Thus, by enabling and promoting efficient, effective, and affordable preventative medicine, next-generation technologies may prove to be the solution to the ballooning costs of healthcare.

Urine analysis has great potential in this respect, considering both its biological richness and its capacity to be a convenient and cost-effective medium for health testing. A large volume of urine is produced daily by the average person: 6–7 urinations totaling 400 to 2000 mL; thus, there is an abundance of samples for collection and analysis.[8] Urine analysis has been used for various diagnostics, including, but not limited to, urinary tract infection (UTI), kidney function, diabetes, pregnancy, and hydration testing.[9] A normal sample of urine consists of several elements: urea, chloride, sodium, amino acids, sulphate, phosphate, potassium, and other trace chemicals, as well as various biomolecules. Abnormal levels of these substances, atypical urine chemical properties, or the presence of certain other chemicals or molecules can be used to diagnose certain health conditions. Analysis of urinary specific gravity and pH can be used to monitor kidney functionality, renal tubular acidosis, hydration, and other urinary tract-related issues. Furthermore, hematuria and proteinuria, the presence of red blood cells and protein in urine, respectively, can be indicators for conditions such as Fabry's disease, nail-patella syndrome, and several others.[9] In addition, certain proteins, ketones, nitriles, bilirubin, or hormones in urine can be possible warning signs for specific conditions and can, therefore, be screened as a preventative care measure. With 1.7% of the United States population suffering from kidney diseases and 9.3% suffering from diabetes — 27.8% of which are unaware of their condition — early screening and diagnosis would enable the patients to receive proper care for these diseases in a timely manner.[10] Particularly for kidney diseases, early intervention enabled by early diagnosis could reduce the risk of cardiovascular complications, kidney failure, or even death, all of which are associated with chronic kidney disease.[11] Additionally, approximately one-in-three women will have at least one UTI by the age of 24 years.[12] Left untreated, or even if treatment is delayed, a UTI can lead to impaired renal function and possibly renal disease. The ability to detect the warning signs for such conditions means starting care earlier, which has the potential to reduce the risk of permanent or life-threatening damage, while also reducing the long-term cost of care.[13,14]

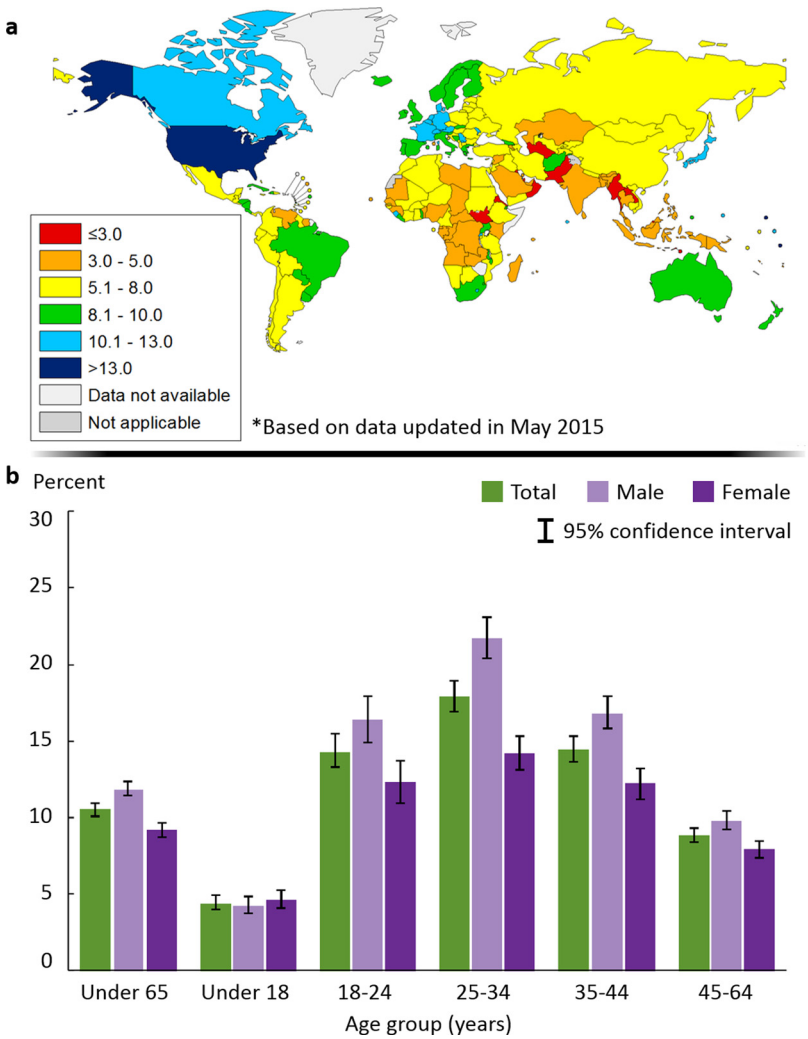

FIG. 1. Healthcare spending and insurance coverage in the world and the United States. (a) Total healthcare expenditure per country as a percentage of each country's respective gross domestic product.[178] W. H. Organization, see http://gamapserver.who.int/gho/interactive_charts/health_financing/tablet/atlas.html for Health Financing: Total expenditure on health as a percentage of the gross domestic product (%): 2014, 2016. Copyright 2017 World Health Organization (WHO). (b) Percentage of individuals in the United States under the age of 65 without health insurance coverage, by age group and sex (2015).[5] Reproduced with permission from Ward *et al.*, see https://www.cdc.gov/nchs/data/nhis/earlyrelease/earlyrelease201605.pdf for Early Release of Selected Estimates Based on Data from the 2015 National Health Interview Survey, National Center for Health Statistics. Copyright 2016 National Center for Health Statistics.

Several recent studies have demonstrated the use of small, efficient, and affordable microfluidic devices as effective analytical tools.[15–17] Moving beyond typical glass- and polymer-based microfluidic devices, paper-based devices offer many of the same diagnostic capabilities in addition to several novel improvements: facile manufacturability, low-cost implementation, and disposability. Despite these advantages, there are notable differences and disadvantages that should also be considered. Paper-based devices, as compared to traditional channel microfluidics, are sometimes limited to simpler samples, shorter separation distances, and have inferior detection limits. Furthermore, prior to analysis using paper-based microfluidics, sample preparation may be required; for instance, certain lateral flow assays require only the supernatant of a centrifuged sample. In addition, optical detection, such as that required by the colorimetric method, is limited in their scope and sensitivity. Other methods of detection, such as electrochemical.[18] (electro)chemiluminescence,[19,20] and metamaterial-based detections,[21] have been demonstrated to improve the sensitivity. With continued research and development, the disadvantages should be remedied.

Herein, we discuss important considerations for point-of-care diagnostic devices. To provide further background information and justification for paper-based urine assays, we discuss what urine is and the theory behind using paper as a platform for the analysis. We then discuss current commercial rapid urine tests and consider where they may fit into the commercial market in the future. Subsequently, we review various fabrication techniques developed for paper-based microfluidic devices. We further present numerous urine assays that are currently used clinically, including their importance to healthcare and clinical diagnosis, with a particular focus on paper-based microfluidic urine assays. We then offer an overview of select biomarker research tailored towards urine diagnostics.

II. CONSIDERATIONS FOR POINT-OF-CARE DIAGNOSTIC DEVICES

In the development of paper-based microfluidic urine analysis devices to provide point-of-care diagnostics, there are regulatory considerations that must be addressed. The U.S. Food and Drug Administration (FDA) sets forth a set of regulatory requirements for diagnostic tests to be used at the point of care or in home. Not only are these requirements law, but they also outline important features that need to be designed when developing any diagnostic test.

The Clinical Laboratory Improvement Amendments (CLIA) of 1988 grants the FDA the authority to evaluate diagnostic devices and tests to determine whether it is "simple" and has an "insignificant risk of an erroneous result."[22] Based on this responsibility, the FDA categorizes diagnostic tests by complexity: waived (least complex), moderate complexity, or high complexity.[23] A test or device is ideally suited for point-of-care diagnostics if it is of low complexity with respect to the following criteria: knowledge; training and experience; reagents and materials preparation; characteristics of operational steps; calibration, quality control, and proficiency testing materials; test system troubleshooting and equipment maintenance; and interpretation and judgement. Herein, we explain the "scorecard" used by the FDA as we assess these criteria in terms of user considerations, sample preparation, user interpretation, and maintenance and quality control.

A. The FDA's "scorecard"

The FDA utilizes a "scorecard" to categorize a diagnostic test by complexity, assigning a score of 1, 2, or 3 for each of the seven criteria listed above.[23] A score of 1 designates the lowest level of complexity, while a score of 3 is the highest complexity. The scores from the seven criteria are added together, at which point a total score of 12 or less is considered moderate complexity, while a score above 12 is considered high complexity. In the case of "simple" devices and tests, the scorecard is not implemented.[22] Instead, the FDA considers a diagnostic device or test to be simple if it fulfils the following qualities: fully automated or self-contained; uses unprocessed samples, including urine; requires only basic sample manipulation, even for decontamination; requires only basic reagent manipulation, such as mixing; analysis does not require intervention; no technical or specialized training necessary; no electronic or mechanical maintenance; produces straight-forward results; operation can be explained via a reference instruction sheet written at no higher than a seventh grade reading level. Diagnostic devices, tests, and procedures that pass this process are those that may be performed by a laboratory or are laboratory examinations and procedures that have been approved by the FDA for home use; furthermore, they are sufficiently simple such that they have an insignificant risk of an erroneous result.[22]

B. User considerations

The ability for users to interact with the device is of utmost importance. Ideally, the device either requires a minimal amount of scientific and/or technical knowledge, or the knowledge that is required to perform tests with the device can be easily acquired at the time of use.[23] Similarly, only a minimal amount of training should be necessary to be able to perform tests with the device. This includes all steps in the testing process: sample and device preparation, the testing protocol, post-test methods, and maintenance between tests. The simpler the test is

and the less training is needed, the less complex the device is considered under CLIA standards. In most cases, paper-based assays provide this simple, straightforward operation consistent with low complexity.

C. Sample preparation

Another important consideration is the complexity of the reagents, materials, and operational steps required for testing. To ensure the accuracy of the test, all reagents and materials involved should be reliable.[23] The reagents should also be stable to ensure the safety of the user and should be prepared appropriately, including the use of packaging and measured quantities. It is also important that the operational steps—sample and reagent measurements, preparation, monitoring, temperature control, timing, and transportation of materials—are straightforward, controllable, or preferably automated. Again, paper-based diagnostic devices address many of these challenges, as the reagents are preloaded onto the paper and operation is often limited to dropping the sample onto the paper.

D. User interpretation

Once the operational steps are completed, the next user-related concern is the interpretation of the results. A minimal degree of interpretation and judgment should be required; the output of the test should be clear and relatively objective.[23] Ideally, quantitative results are reported; however, in the case of qualitative tests, the output must still be straightforward to reduce the risk of an inaccurate interpretation of the results. Paper-based diagnostic devices often provide a simple colorimetric approach to providing results. In some cases, where results are traditionally qualitative, automated readers have been designed to provide more quantitative or definitive results. Such automation can range from image processing software to smartphone applications that utilize the phone's camera and to self-contained devices capable of producing quantitative results; several such readers have been reported for the quantification of paper-based diagnostic test results.[24–28]

E. Maintenance and quality control

Diagnostic tests for the point of care also need to integrate quality control with minimal maintenance requirements. The test should be readily calibrated using reference materials that are stable and accessible; likewise, materials used for quality control must also be stable and accessible.[23] The diagnostic test results should also be compared to those obtained using other devices on a regular basis to ensure that the testing protocols in place are sufficient. Furthermore, any materials needed to perform maintenance must be provided. In the event that there is an issue with the test or device, troubleshooting should be automatic and self-correcting or require only minimal intervention and judgement. Thanks to the stable and disposable nature of paper-based diagnostic devices, there are minimal maintenance requirements and the quality control, consistency, and reliability can be ensured as a part of the manufacturing process.

III. WHAT IS URINE: BACKGROUND AND OVERVIEW

To understand the pertinence of urine in point-of-care diagnostics, it is important to understand what urine is. Urine is a waste material secreted by the kidney and serves as a medium by which the body can eliminate unwanted materials from the blood.[29] Urine is easy to collect, and the fact that large volumes are readily available makes it a convenient material for analysis. Further, urine contains several biological and chemical compounds that may be analyzed as biomarkers for certain conditions.[30]

The waste that is eliminated from the blood via urine includes urea (2% of the total urine excreted), creatinine, uric acid, and ammonia (0.2% combined), and salts and ions (2.8% combined); the other 95% is water leaving the body.[31] Most of the waste materials contained in urine are nitrogenous compounds produced by cellular activity. Specifically, ammonia is produced from the metabolism of amino acids, which is then combined with carbon dioxide in the

liver to produce a less-toxic compound, urea. Furthermore, creatinine is produced when creatine is broken down by skeletal muscle cells to generate the energy necessary for muscle contraction. Lastly, uric acid is the byproduct of nucleic acid breakdown. These compounds circulate through the body via blood before being excreted from the body via blood filtration and urine formation, and subsequent micturition.

Urine is formed in the kidneys (Fig. 2) in stages: filtration, reabsorption, and secretion, followed by excretion.[30,32] Nephrons in the kidney, including the renal corpuscle and the renal tubule, are responsible for urine formation.[32] The first step, filtration, takes place in the renal corpuscles. The renal corpuscle comprises the glomerulus, which is a dense network of capillaries, and Bowman's capsule, which surrounds the glomerulus.[31] Filtration occurs as blood enters the capillaries at a locally high blood pressure, causing plasma, water, dissolved waste products, and small proteins to move out of the capillaries down the pressure gradient and into Bowman's capsule.[30] Important to note is that these capillaries function as a filtration membrane, keeping large proteins and blood cells in the blood while letting other filtrates through; this means that normal urine should be absent of large proteins or blood cells. The collected material is referred to as the renal filtrate, which travels further into the nephron, and continues to the next step of the urine formation process.[30]

The second step of urine formation, reabsorption, occurs in the renal tubule. The purpose of this stage is to preserve any useful solutes and nutrients by recapturing them and returning them to the bloodstream.[32] As the renal filtrate flows through the renal tubule, essential ions, glucose, amino acids, vitamins and nutrients, and small proteins are recollected.[31] The accumulation of these substances in the interstitial fluid within the nephron draws water out from the renal filtrate via osmosis. The water and substances then return to the renal vein via capillaries.

Secretion occurs simultaneously with reabsorption: waste ions, such as sodium, potassium, hydrogen, and calcium, ammonia, creatinine, and other molecules such as drugs move from the blood in nearby capillaries to the interstitial fluid of the kidney, then into the renal filtrate in the renal tubules.[30,31] At this point, the urine formation process is complete. The final renal filtrate leaves the kidneys and is temporarily stored in the bladder, before finally being excreted from the body.

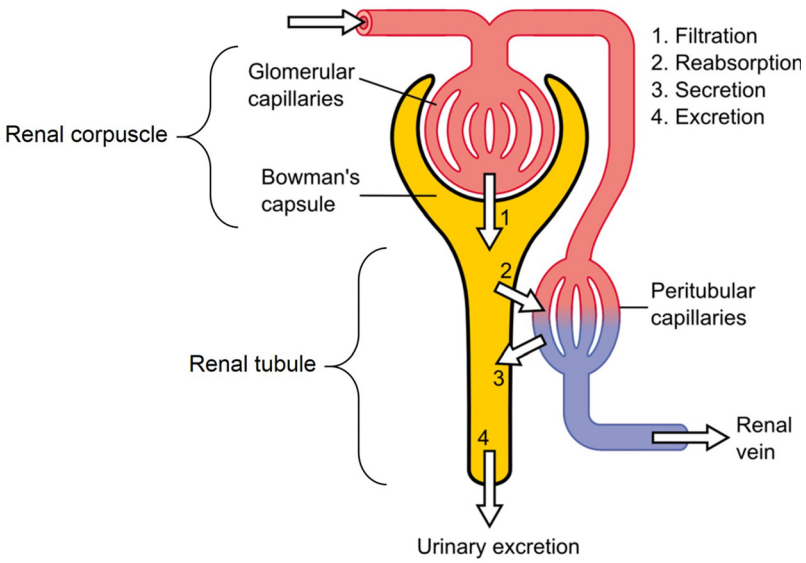

FIG. 2. Nephron physiology for urine formation.[179] Schematic of urine formation, consisting of filtration, reabsorption, secretion, and excretion steps. Reproduced with permission from Boundless, see https://www.boundless.com/physiology/textbooks/boundless-anatomy-and-physiology-textbook/urinary-system-25/physiology-of-the-kidneys-240/tubular-reabsorption-1174-413/ for Tubular Reabsorption. Copyright 2016 Boundless. Licensed under CC BY-SA 4.0/Additional labels added to the original.

As evidenced by the formation process, urine is a direct product of blood filtration and contains several substances that are directly and indirectly related to the health and performance of the body. Urine analysis can be used to measure the levels of normal urine constituents or to detect the presence of abnormal substances which may be indicative of a health condition.[33] Urine analysis tests can be broken down into categories: physical, chemical, microscopic, and bacterial.[32] Physical properties that are commonly observed include color, appearance, volume, specific gravity, and odor. Chemical properties that are often measured include pH, glucose, protein, ketones, bilirubin, urobilinogen, blood, nitrites, and ascorbic acid. Furthermore, urine can be examined under the microscope to observe the presence of cells, crystals, bacteria, or other components.

IV. WHAT IS PAPER: THEORETICAL STUDIES

Another important aspect of paper-based point-of-care devices is how paper enables fluid transport as the substrate. To manufacture multifunctional, highly sensitive, and stable paper-based diagnostic devices, precise control of fluid flow is necessary during the hydrophobic barrier patterning, reagent deposition, and sample processing.[34,35] In conventional PMDS chips or 3 D-printed chips, the flow is often characterized by the Hagen–Poiseille law assuming pressure-driven flow. On the other hand, the flow in paper-based microfluidic devices is a passive process and governed by capillary fluid transport. Capillary action is an outcome of the interplay between adhesive and cohesive forces and is driven by intermolecular forces between the fluid particles at the liquid-air interface (i.e., surface tension, a cohesive force) and at liquid-porous fiber interface (i.e., van der Waals force, an adhesive force).[34,36] Therefore, the flow rate through a paper channel can be controlled by manipulating the cross-sectional area, permeability of the paper, channel length, or fluid viscosity.[34,35] In the following sections (Sections IV A – G), wet out and fully wetted flow in both constant and varying-width channels is summarized. Moreover, the effects of evaporation, relative humidity, and hydrophobic barriers on the wicking speed are discussed.

A. Types of paper and other porous substrates

Cellulose-based materials, such as paper and nitrocellulose membranes, are commonly used as the substrate for point-of-care diagnostic devices. Considering a lateral flow assay—a device consisting of a sample pad, a conjugate pad, a reaction zone with test indicator lines or symbols, and an absorbent pad, all of which are connected via a backing membrane that facilitate flow of the sample from the sample pad to the absorbent pad—as an exemplary paper-based diagnostic device, multiple materials can be used as a substrate.[37] The sample pad, where the fluid sample is applied to the device, may be made of cellulose paper or glass fiber, as these offer continuous and homogenous flow of the sample. The conjugate pad, where biorecognition molecules are dispensed, must be able to release the labeled conjugate upon contact with the fluid sample. Accordingly, the conjugate pad is often made of cellulose, glass fiber, or polyester. To connect the different regions of the assay, a nitrocellulose membrane may be used.

Nitrocellulose membranes are available in a variety of configurations. The pore size may vary from as little as 0.1 μm to 0.45 μm, where larger pore sizes are more compatible with larger molecules.[38] Similarly, cellulose papers are categorized into different grades.[39] For example, grade 1 cellulose chromatography paper has the highest linear flow rate (when the fluid is water). Grade 2 has a slower flow rate, but is well-suited for higher resolution, particularly when optical scanning will be used.

An example of a non-paper, porous substrate is cotton, which is similarly a fiber-based material. Cotton is a natural choice for devices which require absorption, such as lateral flow assays. Cotton can also be designed such that the fluid channel consists of hydrophobic and hydrophilic regions in the surface layers and the interior, respectively.[40] Cotton is so absorptive, however, that when the fluid sample travels through it, the concentration of components within the sample dramatically decreases; the dilution effect may also be a function of the distance from the sample input to the readout zone.

B. Paper wet out

1. Constant-width channels

Washburn's equation is a model for simple imbibition and was derived by combining capillary theory with Hagen–Poiseuille flow. It can be used to predict the fluid front (L) in a one-dimensional porous media. Here, the substrate is assumed to be a bundle of capillary tubes with a non-limiting reservoir and a constant cross-sectional area throughout the length [Eq. (1)]

$$L = \sqrt{\frac{\gamma D \cos\theta}{4\mu} t}, \tag{1}$$

where D, γ, μ, and θ are the average pore diameter, the effective surface tension, the viscosity, and the capillary contact angle, respectively. It can be seen that the fluid front is proportional to the square root of time, t. Although the characteristics of the paper may cause flow that is inconsistent with this equation, the equation is frequently used as a first-order approximation because of the good empirical description of the fluid front and the ease of use. In this equation, it is assumed that paper is a matrix with homogeneous pore size, constant cross-sectional area, no impurities, and consistent hydrophobic channels which do not exert a capillary force. The average pore diameter of a paper substrate can be estimated by rearranging this equation and substituting experimental data. This equation is used under the viscous, incompressible, laminar, and saturated conditions without considering evaporation.[34,41,42]

2. Constricted flow

If we consider two connected channels with different widths, the non-limiting fluid-reservoir assumption will be violated if the narrower channel comes first [Fig. 3(a)]. In this case, as the fluid migrates through the wider channel, the velocity of the fluid flow decreases due to the conservation of mass along the channel; as the cross-sectional area of the channel increases, the velocity of fluid flow decreases such that the flow rate remains constant. Employing the transport time of a sample or a reagent as an adjustable parameter is a practical application of constricted flow.

3. Abundant flow

Abundant flow happens when a fluid migrates through a wide channel into a narrower one [Fig. 3(a)]. In this case, the assumption of a non-limiting reservoir is valid, and the change in channel width does not affect the flow rate because sufficient fluid is supplied to the transition point. Experimental data show that the flow from the wider channel can act as a non-limiting reservoir assuming that the channels are equal in depth.

C. Fully wetted flow

1. Constant-width channels

Darcy's law (1856) can be used to describe fluid flow through a pre-wetted channel in porous media. This equation, which is based on the Navier–Stokes equations, represents the fluid flow through a straight, constant-width channel

$$Q = -\frac{\kappa WH}{\mu L}\Delta p, \tag{2}$$

where Q, μ, and κ are the volumetric flow rate, viscosity of the fluid, and the permeability of the paper, respectively; WH and Δp are the area of the channel perpendicular to flow and the pressure difference along the flow direction over the length, L, respectively. Other assumptions in Darcy's law include a circular cross-section of fibers, straight capillaries, and constant fluid

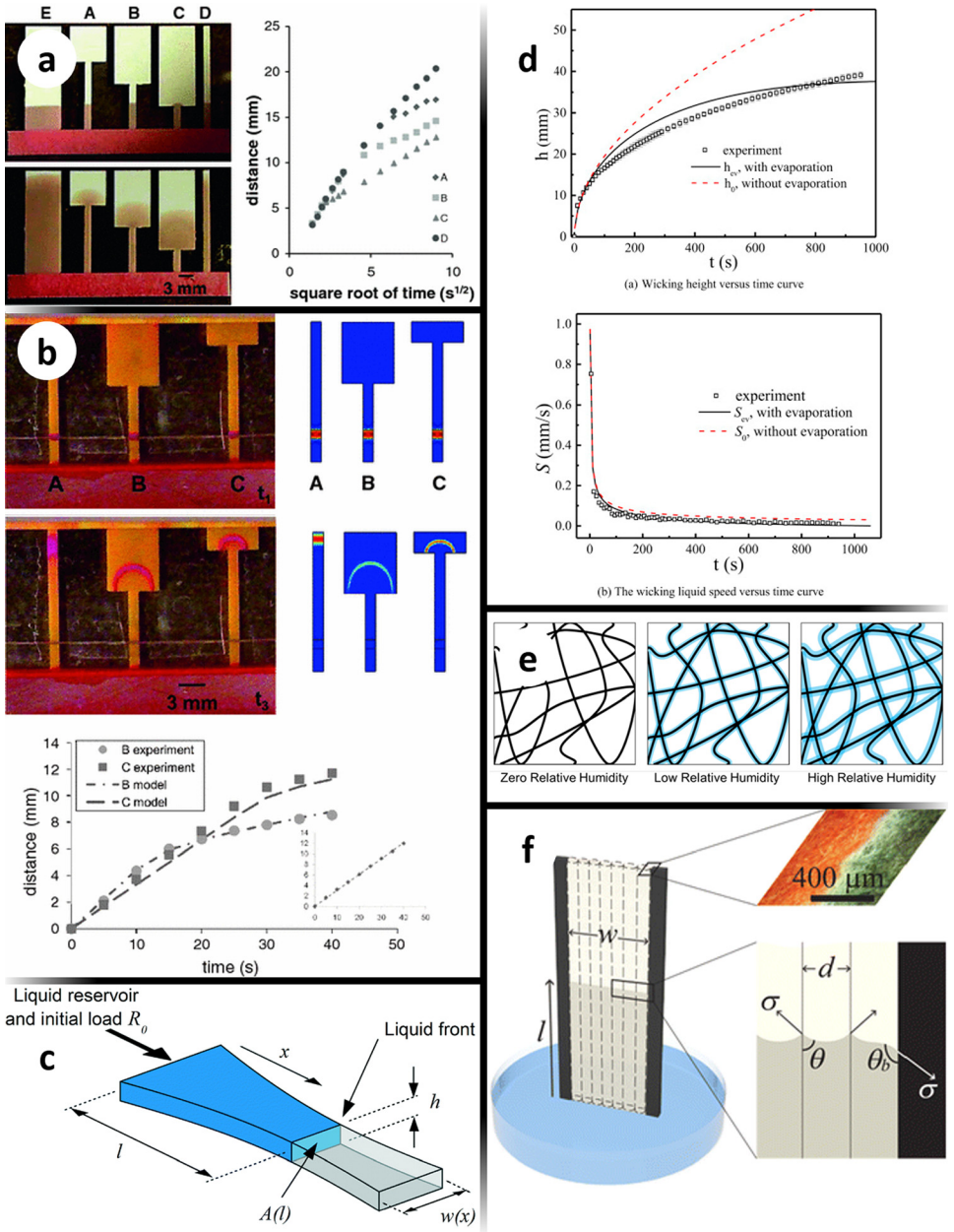

FIG. 3. Experimental and theoretical examples of flow through a paper-based substrate. (a) Wet-out fluid flow for uniform and non-uniform channels.[180] The upper image shows equal fluid transport for all geometries initially, while the lower image shows the change is transport speed relative to the channel width. Wider regions have slower fluid transport to the greater cross-sectional area and the conservation of mass. Plot (at right) displays the distance traveled by the fluid front vs. the square root of time for strips A through D. Reproduced with permission from Fu *et al.*, Microfluid. Nanofluid. **10**(1), 29–35 (2011). Copyright 2010 Springer-Verlag. (b) Experimental and theoretical modeling results for fluid flow in fully wetted strips of varying widths and geometries.[180] Reproduced with permission from Fu *et al.*, Microfluid. Nanofluid. **10**(1), 29–35 (2011). Copyright 2010 Springer-Verlag. (c) Schematic representation of the flow domain for fluid flow through an arbitrary cross-section.[44] Reproduced with permission from Elizalde *et al.*, Lab Chip **15**(10), 2173–2180 (2015). Copyright 2015 Royal Society of Chemistry. (d) Illustration of water absorbed and retained by the cellulose fibers in unwetted paper, dependent on humidity.[42] Above, a plot representing the fluid front travel distance vs. time; below, a plot showing the fluid travel speed vs. time. Reproduced with permission Liu *et al.* Appl. Thermal Eng. **88**, 280–287 (2015). Copyright 2014 Elsevier Ltd. (e) Effect of humidity on imbibition in paper channels.[41] For all papers included, there is a linear trend. Reproduced with permission from Castro *et al.*, Microfluid. Nanofluid. **21**(2), 21 (2017). Copyright 2017 Springer Berlin Heidelberg. (f) Schematic of a paper channel with wax (hydrophobic) boundaries.[46] Paper is assumed to consist of stacked capillaries. The contact angle within/between the capillaries is less than 90°, while the contact angle adjacent to the boundary is greater than 90°, resulting in reduced capillary driving force along the boundaries. Reproduced with permission from S. Hong and W. Kim, Microfluid. Nanofluid. **19**(4), 845–853 (2015). Copyright 2015 Springer Berlin Heidelberg.

properties.[34,43] The fluid front migrates a certain distance though the constant-width channel in time t, which can be expressed as follows:

$$t = \frac{V}{Q} = \frac{V\mu L}{\kappa W H \Delta p} = \frac{\mu L^2}{\kappa \Delta p}, \tag{3}$$

where V is the volume of the fluid at time t. Δp is assumed to be constant because it is governed by the capillary force. When the permeability of the paper is constant, flowing time in a constant-width channel only depends on L.

2. Varying-width channels

If the width of a channel varies, Darcy's law can be applied again to determine the volumetric flow rate [Fig. 3(b)]. It can be assumed that equal volumetric fluxes are imposed in serially connected straight channel segments with different widths

$$Q = -\frac{\Delta \mathrm{p}}{\left(\frac{\mu}{\kappa}\sum_{i=1}^{N}\frac{L_i}{W_i H_i}\right)}, \tag{4}$$

where $W_i H_i$ is the area of the channel segment perpendicular to flow. L_i and Δp are the length of segment i in the direction of flow and the pressure difference across the length of the channel, respectively. By using Ohm's law analogy, the total volumetric flux through a paper strip with N segments in series and parallel can be estimated. In this approach, Δp (pressure difference across the length of the channel) and Q (flow rate through the channel) are the fluidic counterparts to voltage change and current, respectively. κ is the fluid permeability of the paper. The fluidic counterpart to electrical resistance is given by

$$\text{“}\textit{fluidic resistance}\text{”} = \frac{\mu L_i}{\kappa W_i H_i}. \tag{5}$$

In series channels, the total resistance in a string of fluidic components is the sum of each of the individual fluidic resistances; in parallel channels, the resistance is the reciprocal of the sum of the reciprocals of the individual resistances.[34]

D. General equation for wicking speed in paper of arbitrary cross-section

A general equation for the wicking speed through a piece of paper with an arbitrary cross-section can be derived by using the continuity and momentum equations under the Stokes regime (assuming isothermal conditions and neglecting gravity), where v is the average fluid velocity, μ is the fluid viscosity, k is the permeability of the porous substrate, and p is the average pressure[44]

$$\nabla . v = 0, \tag{6}$$

$$v = -\frac{k}{\mu}\nabla p. \tag{7}$$

Integrating Eq. (7) and substituting $Q = v(x)A(x)$, where Q is flow rate, p_0 is the pressure at x = 0, and p_c is the average capillary pressure at the fluid front,

$$p_0 - p_c = Q\frac{\mu}{k}\int_0^l \frac{dx}{A(x)}. \tag{8}$$

By adding the above equations and defining $\Delta p = p_{atm} - p_c$ as the change in pressure where p_{atm} is atmospheric pressure, the following equation for the wicking velocity, v, as a function of l can be derived

$$v(l) = \frac{\Delta p}{A(l)\left[R_0 + \frac{\mu}{k}\int_0^l \frac{dx}{A(x)}\right]}, \tag{9}$$

where $v(l)$ is the fluid front velocity and $A(l)$ is the corresponding cross-sectional area of the fluid front, and R_0 is the flow resistance. By substituting $v(l) = \frac{dl}{dtv}$ and integrating Eq. (9), an implicit expression is derived to approximate the position of the fluid front

$$\frac{kR_0}{\mu}\int_0^l A(l')dl' + \int_0^l \left[A(l')\int_0^{l'} \frac{dx}{A(x)}\right]dl' = Dt, \tag{10}$$

where $D = k\Delta p/\mu$ is a diffusive coefficient (m^2/s). This equation can also be used to predict the time needed for filling a piece of paper with an arbitrary cross-section, A, and length, l [Fig. 3(c)]. It should be noted that this equation is valid under the assumption of constant contact angle and constant curvature of the air–liquid interface during the imbibition.

E. Effect of evaporation on wicking speed

Liu *et al*. introduced a model to predict the fluid front by considering evaporation.[42] The wicking liquid mass was used to predict the height of the wicking fluid front, h_{ev} (mm), as follows:

$$h_{ev} = 2Ne^{-Mt}\int_0^{\sqrt{t}} e^{Mt^2}dt, \tag{11}$$

where

$$M = \frac{2m^*_{ev}}{\rho\varepsilon\delta} \quad and \quad N = \sqrt{\frac{\sigma\cos\theta}{\mu}\frac{K}{\varepsilon R}}$$

with m^*_{ev}, ρ, ε, and δ are the evaporation rate ($mg/(m^2s)$), density (kg/m^3), effective porosity, and thickness, respectively; and R, μ, σ, θ, and K are the effective pore radius (μm), viscosity (Pa*s), interfacial tension (N/m), contact angle, and permeability (m^2), respectively.

Consequently, the wicking speed, S_{ev} (mm/s), can be predicted as

$$S_{ev} = \frac{dh_{ev}}{dt} = N.t^{-\frac{1}{2}} - 2M.Ne^{-Mt}\int_0^{\sqrt{t}} e^{Mt^2}dt\,. \tag{12}$$

Experiments were performed in a closed room with the following assumptions to demonstrate that the model agrees with the experimental data: the air flow is equal to zero, the relative humidity is 52%, the water saturation pressure is 2338 Pa, and the latent heat of vaporization of water is 2454.3 kJ/kg [Fig. 3(d)]. This assumption led to the constant evaporation rate equal to 0.0407 $g/m^2{\cdot}s$.

F. Effect of relative humidity on wicking speed

Based on the Washburn equation, any channel width will have the same imbibition behavior since the effective material pore size and the liquid properties are the only parameters which have an effect on the liquid front. However, this is invalid for non-ideal laboratory settings,

such as imbibition in different relative humidity. This issue revealed the need of investigating the effect of these parameters on fluid flow.

Fries *et al.* has presented a model by considering the evaporation effect, where y_f is the imbibition distance of the liquid front[41,45]

$$y_f = \left[\frac{a - a\exp(-2bt)}{b}\right]^{\frac{1}{2}}, \tag{13}$$

where

$$a = \frac{K\gamma\cos\theta}{D_e\phi\mu} \quad \text{and} \quad b = \frac{F(W+T)}{\phi\rho WT}$$

with D_e, K, and ϕ being the effective pore diameter, the effective permeability, and the porosity of the material, respectively; and F is the evaporation flux, W is the width of the channel, ρ is the liquid density, and T is the paper thickness, respectively.

Since paper naturally absorbs moisture at a high relative humidity, Castro *et al.* developed a modified version of the Fries *et al.* model. This model takes into consideration the residual water associated with the relative humidity.[41] Neglecting the gravitational effect, it can be simplified to

$$y_f = \left[\frac{a - a\exp(-2bt)}{b}\right]^{\frac{1}{2}}, \tag{14}$$

where

$$a = \left(\frac{K}{2\phi}\right)^{\frac{1}{2}}\frac{\gamma\cos\theta}{\mu} \quad \text{and} \quad b = \frac{F(W+T)}{\phi\rho(1-S_w)WT}$$

with S_w being the degree of water saturation. The Washburn model can be represented in a similar form to other models for the purpose of comparison

$$y_f = \sqrt{\left(\frac{K}{2\phi}\right)^{\frac{1}{2}}\frac{\gamma\cos\theta}{\mu}t}. \tag{15}$$

By increasing the relative humidity, the difference between the three mentioned models decreases due to decreased evaporation [Fig. 3(e)]. In the case of no evaporation (F = 0), both Eqs. (13) and (14) reduce to Eq. (15) (the Washburn model).

G. Effect of hydrophobic barriers on dynamics of fluid flow

To study the effect of boundaries on flow dynamics in paper, wax-bounded capillary channels were considered in a case study by Hong and Kim.[46] It was observed that the hydrophobic material partially comprises the inner surface of the capillary channels adjacent to the side boundaries, which leads to a higher contact angle at the side boundaries (θ_b) compared with that occurring in bulk capillaries [Fig. 3(f)]. Therefore, the modified force balance for a control volume can be expressed as

$$(\pi d\sigma\cos\theta - 8\pi\mu ll')\left(\frac{w}{d}\phi^{\frac{1}{3}}\right)\left(\frac{b}{d}\phi^{\frac{1}{3}}\right) + \beta\pi d\sigma\cos\theta_b\left(\frac{b}{d}\phi^{\frac{1}{3}}\right) = 0, \tag{16}$$

where β is the length of the advancing contact lines in contact with the wax boundaries and should be experimentally determined; w, ϕ, b, and d are the channel width, the porosity, the

thickness of the paper, and the pore diameter, respectively; and l' is the first derivative of l with respect to time, which is the imbibition length, with respect to time. By solving Eq. (16), the capillary imbibition through a channel with wax boundaries can be modeled by

$$l_m(t) = k\sqrt{\left(1 + \beta\frac{d}{\phi^{\frac{1}{3}}w}\frac{\cos\theta_b}{\cos\theta}\right)\frac{\sigma}{\mu}t}, \tag{17}$$

where l_m is the imbibition length, k is a proportionality constant introduced to take into account the geometry of the pores in porous media and depends on pore diameter and contact angle, σ is the surface tension, and μ is the dynamic viscosity.

This model has been shown to agree with experimental data of capillary flow through paper channels with a variety of boundary forms and offers a simple way for controlling the wicking speed in paper channels by considering the effect of the wax boundaries. Moreover, other approaches have been proposed by other researchers for controlling the wicking speed in paper-based microfluidics, as reviewed in Ref. 47.

V. APPROACHES TO RAPID PAPER-BASED DIAGNOSTICS

Up to this point, this review has provided the theoretical background for paper-based microfluidic devices for urine analysis. It is also necessary to the consider current approaches to paper-based diagnostics. Commercially produced, rapid tests for urine analysis are readily available in the market today [Fig. 4(a)]. These paper-based devices rely on the function of paper and the characteristics of urine, as described above. Dipsticks, test strips, and lateral flow assays are popular among consumers due to their low cost, easy-to-read qualitative and semi-quantitative measurements, small size, portability, and ease of use. However, the limited quantitative precision and limit of detection restricts their use to screening purposes.[48] Paper-based microfluidic devices are abundant in the form of test strips for detecting chemical reagents and

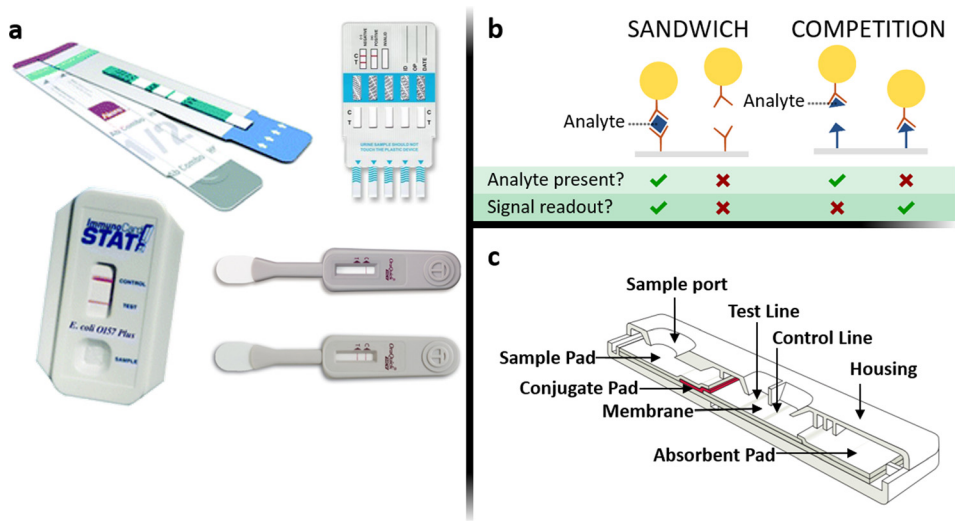

FIG. 4. Commercial rapid test examples with lateral flow assay highlighted. (a) Clockwise from upper-left: Determine™ HIV 1/2 Ag/Ab Combo.[34] Adapted from Yetisen *et al.*, Lab Chip **13**(12), 2210–2251 (2013). Copyright 2013 The Royal Society of Chemistry. 5 Panel Drug Test Kit.[181] (Image from HomeHealthTesting.com. OraQuick: HCV Rapid Antibody Test.[182] Copyright Image copyright OraSure Technologies, Inc. All rights reserved. Used with permission. ImmunoCard STAT!® E. coli O157 Plus.[34] (b) General comparison of the functionality of direct (sandwich) and competitive lateral flow assays.[183] Reproduced with permission from nanoComposix, see https://nanocomposix.com/collections/bioready-nanoparticles for BioReady Nanoparticles for Lateral Flow. Copyright 2017 nanoComposix (c) Example of a lateral flow assay, displaying the sample pad where the sample is loaded, the conjugate pad containing the reactive molecules, the test and control lines on the membrane, and the absorbent pad which provides capillary force to draw the fluid sample through the device.[34] Adapted from Yetisen *et al.*, Lab Chip **13**(12), 2210–2251 (2013). Copyright 2013 The Royal Society of Chemistry.

lateral flow assays for biological tests. As research continues and lower-cost manufacturing is developed and broad applications are introduced, they are likely to become more ubiquitous in the clinical field. The predominant forms of commercial rapid tests and various methods of reading the results are summarized herein.

A. Dipsticks and test strips for chemical analysis

One of the most common types of commercially produced, rapid urinary tests, urine dipsticks are designed for routine qualitative analysis of urine samples. Generally, they are dipped into a urine sample, then undergo a chemical reaction with the components of the urine to show the result, which is often in the form of a color change. They are largely accepted in clinical use due to their facile operation and sufficient accuracy.[49–52] Furthermore, the low-cost nature of commercial test strips is largely due to the mass production techniques employed in fabrication.

Originating in the 1950 s from the paper-based dipstick for glucose quantification for diabetic individuals, dipsticks were first made commercially available in the 1960 s.[53] Nowadays, dipsticks and test strips have evolved significantly but retain their notably simplistic operation. Utilizing the colorimetric method, most urinary dipsticks display a color which can directly correlated to the concentration of the substance of interest by visual comparison with a color chart.[54,55] The test strips can be used for single-substance identification or can be used to identify multiple substances simultaneously as multiplexed strips.[56] These strips are available for testing various substances, including specific gravity, acidity/alkalinity, urinary protein, glucose, ketones, blood and blood components, including hemoglobin, nitrates, drugs, and metabolites.[57]

Specially designed readers, such as the Urisys 1100® Urine Analyzer, allow for more quantitative results of the colorimetric sensors compared with qualitative results of visual interpretation.[58,59] Devices, including the Urisys 1100® by Roche Diagnostics, the cobas® u 411 analyzer also by Roche Diagnostics, and the LAURA® Semi-Automated Urine Strip Reader by Erba® Mannheim, rely on a reflectance photometer to optically examine the test strips.[60–62] These devices, while serving the purpose of point-of-care diagnostics, tend to be rather bulky. Beyond the colorimetric (optical) method, there are other means of analyte detection which are commercially available, including electronic strip readers which directly provide the user quantitative results. Kaiwood Technology and Otsuka Electronics are two exemplary companies who have developed strip readers which utilize charge-coupled device imaging and photo-diode scanning, respectively.[57] Despite these advances and the wide applicability of test strips, they are limited in their detection range and precision and are therefore restricted to use for screening purposes, as opposed to true diagnostics.

B. Lateral flow assays for other biomarkers

In addition to the dipstick format of test strips, another very common commercially produced urine-testing technology is the lateral flow assay. Lateral flow tests date back to the 1950 s, as well, when latex agglutination assays and radio-immunoassays were introduced.[63,64] Later in the 1970 s, nitrocellulose membranes were adapted for use beyond filtration, as they were shown to act as a suitable substrate for molecular detection.[65–68] In the following decade, serological lateral flow tests were developed and came to market, most notably in the form of urine-based pregnancy tests.[69,70] Additionally, lateral flow assay urine tests are commercially available for ovulation and menopause.[71,72]

There exists a great diversity of lateral flow assays offered in various sizes, shapes, and configurations. The assay may be in a standalone form or may be encased in a plastic housing to improve its durability and ease of handling. Some lateral flow assays even include a digital readout screen as an improvement on the conventional symbolic result appearing on the lateral flow assay itself. Multiplexing can be achieved with a configuration similar to that of the dipstick through either multiple channels stemming from the same sample input, or through multiple analytic detection zones on the same device.[34]

Lateral flow assays can be categorized into two major types: direct and competitive formats [Fig. 4(b)]. Direct assays take the form of double-antibody sandwich assays, in which the response observed on the test line of the assay is directly proportional to the concentration of the analyte under investigation.[73] Competitive assays utilize inhibitive antibody binding to the analyte, by which the test line response is inversely proportional to the analyte concentration.[73] In comparing these two formats of lateral flow assays, it is important to note that for small analytes and in cases when an antibody has been raised against only one site on the molecule, the competitive assay is necessary. A similar yet far less popular assay is the flow-through vertical format. The lack of popularity is a result of the multi-step execution: the nature of this assay requires that the user perform sample loading, washing, and addition of the conjugates.[74]

Lateral flow assays usually consist of a sample pad, a conjugate pad, a reaction zone with test indicator lines or symbols, and an absorbent pad, all of which are connected via a backing membrane that facilitates the flow of the sample from the sample pad to the absorbent pad [Fig. 4(c)].[75] At the sample pad, the test sample is loaded onto the device, and a buffer solution is added, if necessary, to promote capillary flow of the sample through the device. Once the sample reaches the conjugate pad, the target molecules (i.e. the analyte of interest) bind specifically to the preloaded antibodies, which then continue to flow to the reaction zone. In the reaction zone, reactions in the test line and control line cause observable color changes, which can be interpreted as the presence or absence of the analyte of interest. Finally, the absorbent pad on the far end of the device acts as a sink for the sample to maintain the fluid flow.

Lateral flow assays are generally affordable, compact, and easy to use. In the case of urine analysis, there is minimal to no sample preparation required, further increasing the user-friendliness. Furthermore, they can be multiplexed to test for multiple analytes in a single sample on one device. However, they also suffer from the same weakness as dipsticks: a lack of sensitivity. Quantitative reading devices, similar to those for dipstick rapid tests, exist but are often not accessible for consumer use, particularly in low-income developing nations.

Thus, methods of improving the sensitivity of lateral flow assays, behind simply using a reading device, is a topic of recent interest in the field (Fig. 5).[76] For instance, a fluorescent tag can be added to the antibody for optical signal enhancement to facilitate more sensitive reading.[77] Similarly, enzyme enhancement involves the addition of enzymes to the antibody such that every bound antibody in the reaction zone produces many colored molecules.[78] Another colorimetric approach is polymerization-based amplification.[78] This method uses a photoinitiator and a monomer solution to form a hydrogel around the analytes of interest; when a base is added to the sample, a pH indicator in the hydrogel changes color to produce a visual result. Another form of optical enhancement is dual gold nanoparticle (AuNP)-conjugate-based signal amplification.[79] The AuNPs bind to the analyte and increase the contrast between the collected analyte in the reaction zone and the lateral flow assay's background. Similarly, silver can be deposited in a layer on top of the AuNPs to further increase the visibility of the analyte. As an alternative to optical enhancement, thermal contrast can be employed to detect the presence of analytes on the test and control lines, which offers greater precision compared with some optical methods.[80] Another notable method for improving the sensitivity of lateral flow assays is the application of surface plasmonic resonance (SPR).[81] The SPR method relies on the differential refraction of light on the surface of the lateral flow assay; analytes on the surface are detected based on the changes in the light refraction pattern.

C. Micropads (μPads)

Micropads, also known as μPads, represent a microfluidic approach to rapid paper-based diagnostics. While not yet commercially produced or sold, extensive research on micropads demonstrates their potential.[34,82] The channels and detection zones are delineated by hydrophobic boundaries, which may be formed by various fabrication techniques, as described in Sec. VII B. Micropads take advantage of the capillary force of paper to draw fluidic samples through the formed channels.[76] Detection zones, which are interspersed between channels or as standalone features, can be preloaded with chemical reagents or biological reagents for rapid analysis

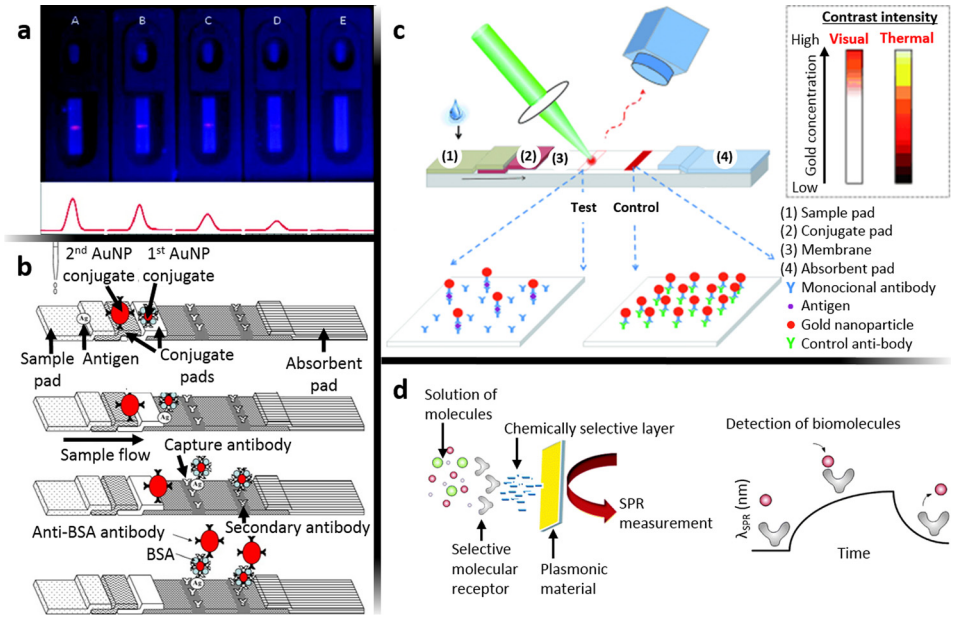

FIG. 5. Methods to improve the sensitivity of lateral flow assays. (a) Fluorescence-based signal enhancement by addition of fluorescent proves.[77] Reprinted with permission from Li *et al.*, Anal. Chem. **82**(16), 7008–7014 (2010). Copyright 2010 American Chemical Society. (b) Dual gold nanoparticle (AuNP) conjugate-based signal amplification, which causes larger aggregates of the analyte at the control and test lines to improve visibility (note, silver can be deposited on top of the AuNPs to further enlarge the analytes).[79] Reproduced with permission from Choi *et al.*, Biosens. Bioelectron. **25**(8), 1999–2002 (2010). Copyright 2010 Elsevier B.V. (c) Thermal contrast imaging to provide a quantitative readout of the presence of an analyte at the control and test lines.[80] Reproduced with permission from Qin *et al.*, Angew. Chem. Int. Ed. **51**(18), 4358–4361 (2012). Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (d) Surface plasmonic resonance (SPR) for analyte detection due to analytes on the surface causing changes in the refractive index of the material.[81] Reproduced with permission from Couture *et al.*, Phys. Chem. Chem. Phys. **15**(27), 11190–11216 (2013). Copyright 2013 Royal Society of Chemistry.

of samples. For such chemical tests, the colorimetric method is often employed for visualizing the results. A limitation in this application of the colorimetric method is the coffee ring effect—sample, reagents, and resulting color change often expand and settle to the outer edge of the defined zone, causing non-uniform color distribution.[83] Further, electrochemical devices have been developed, which utilize conductive inks that undergo electrochemical reactions in the presence of an analyte.[84] For such electrochemical tests, resistance and conductance values are measured to detect these changes in electrochemical properties as an indicator of the sample of interest.

Micropads reap the same benefits as other paper-based diagnostic devices as well as those of conventional microfluidic devices. Attributed to their paper-based nature, they are low cost and easy to manufacture, and are disposable. At the same time, they provide many of the same point-of-care analytical capabilities of traditional glass- and polymer-microfluidic devices, without the need for pumps, a power supply, or extensive sample preparation.

D. Additional approaches to paper-based diagnostics

Recent advances in paper-based microfluidics and urine analysis pave the way for the future of point-of-care urine diagnostics. In addition to test strips, lateral flow assays, and micropads, which represent the most commonly investigated forms of paper-based diagnostics, there are other approaches to rapid paper-based diagnostics that cannot be placed into these categories. For example, Piety *et al.* recently reported a low-cost paper-based screening test for sickle cell anemia.[24] A droplet of blood mixed with a buffer is deposited with a reducing agent onto paper and then diffuses through the paper by capillary force, leaving behind unique blood staining patterns that can be interpreted as a diagnostic indicator.

VI. EMERGING TECHNOLOGIES FOR READING PAPER-BASED ASSAYS

For the aforementioned paper-based assays, there are various methods for reading the results that have been proposed as more sensitive alternatives to the readers which are currently available. The following is an overview of recently proposed, next-generation methods for reading the results of paper-based assays.

Colorimetric test strips are commonly interpreted by the naked eye, providing semi-quantitative results [Fig. 6(a)].[54,55] Optical readings can provide quantitative results.

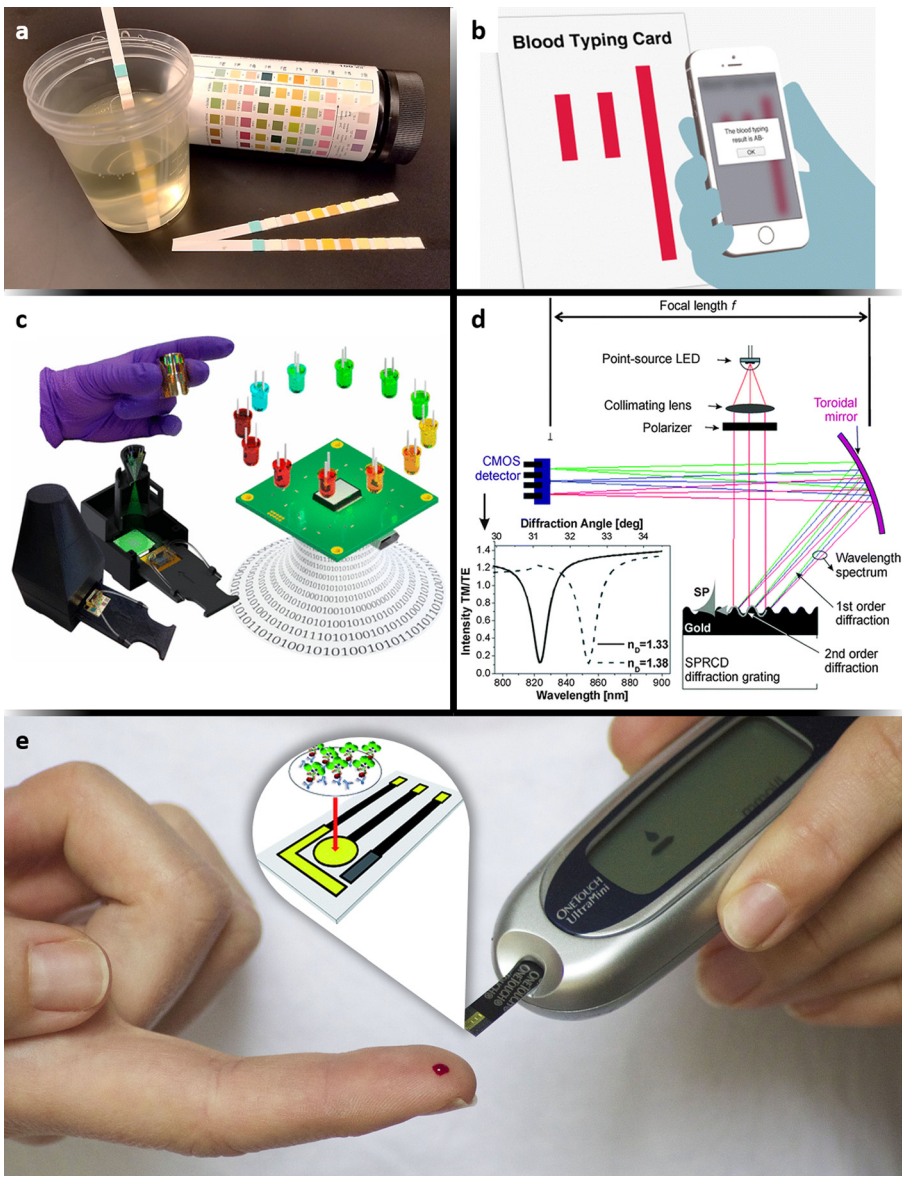

FIG. 6. Various methods for reading quantitative detection of analytes. (a) Visual, naked eye-based analysis using calibrated colorimetric chart. Photograph shows 10 Parameter + ASC URinanlyss Reagent Strips by Xlar. (b) Smartphone-based reading using custom application and camera.[83] Reprinted with permission from Guan *et al.*, Anal. Chem. **86**(22), 11362–11367 (2014). Copyright 2014 American Chemical Society. (c) Computational sensing using mobile light-based plasmonic chip sensors.[84] Reprinted with permission from Ballard *et al.*, ACS Nano **11**(2), 2266–2274 (2017). Copyright 2017 American Chemical Society. (d) Surface plasmonic resonance sensing apparatus utilizing special diffraction grating structure to couple light onto a surface plasmon and disperse the diffracted light for spectral readout using a charge-coupled device camera.[184] Reproduced with permission from Piliarik *et al.*, Biosens. Bioelectron. **24**(12), 3430–3435 (2009). Copyright 2008 Elsevier B.V. (e) Electrochemical-based reading of analytes using electrodes and handheld device.[87,185] Reproduced with permission from Tang *et al.*, Anal. Methods **6**(22), 8878–8881 (2014). Copyright 2014 Royal Society of Chemistry; background image licensed under CC0 Creative Commons.

Specifically, smartphone-based systems have been proposed, which rely on the phone's built-in camera and a custom application to capture an image of the reaction area on a paper-based device, and process the image to interpret the color as the amount of analyte present [Fig. 6(b)].[24–28,85] Beyond optics-based methods, other methods and devices for reading paper-based assay results have been proposed. Machine learning can be leveraged to develop advanced sensing devices capable of providing the same results as larger, more expensive instruments in a portable, low-cost design. For instance, mobile plasmonic sensors coupled with machine learning were implemented in a computational sensing device to provide digital information about the inserted sample using light-emitting diodes instead of bulky and expensive stabilized light sources or high-resolution spectrometers[86] [Fig. 6(c)]. Full-scale SPR can also be measured to quantify the results of paper-based assays, as briefly described for lateral flow assays [Fig. 6(d)]. This method interprets the refraction of light from the paper's surface, and analytes on the paper surface cause a change in the refractive index, which leads to a change in the plasmonic wavelength of the excitation angle. In a novel approach, the refracted light we manipulated in a way that it could be captured by a charge-coupled device camera for a spectral readout.[81] Another reading method, which was briefly referenced for test strips and dipsticks, is to measure the electrochemical properties resulting from a reaction with the analyte of interest [Fig. 6(e)]. A paper substrate with three printed electrodes—a working electrode, a reference electrode, and a counter electrode—can be inserted into an electrochemical reader to interpret the presence of analytes by applying a voltage and measuring the resulting current.[87,88]

VII. FABRICATION TECHNIQUES

In this section, we review the fabrication techniques used for each type of paper-based diagnostic device, with a particular focus on the various methods reported and developed for paper-based microfluidic devices. While large-scale manufacturing methods for test strips and lateral flow assays are well-established for commercial sale, the fabrication of micropads is still under active development. Commensurate among all the paper-based diagnostic devices described here is a permeable wicking substrate—which may be paper, cotton, nitrocellulose membrane, etc.—on which chemical and biological reagents, physical and chemical boundaries and features are added.

A. Established fabrication methods for commercially available paper-based devices

1. Fabrication of test strips

Test strips consist of a backing material, testing zones which may be part of the paper backing, and chemical reagents impregnated in the testing zones that react once in contact with a sample. The fabrication of commercial test strips is simple, involving a single soaking step.[76] Taking pH test strips as an example, a piece of filter paper is soaked in a mixture of acid-base indicators.[76] Once the paper is dry, detection reagents are added that change color depending on the reaction of the indicator with the analyte, which, in this case, is hydrogen ions. Similar processes are used in test strips for other substances, often with the addition of color indicators.

2. Fabrication of lateral flow assays

The generalized fabrication of commercial lateral flow assays consists of five steps: assembly of components, dispensing of biological reagents, drying, cutting to proper size, and lamination or encasing.[76] First, the sample pad and absorbent pads are cut, and then, the sample pad, absorbent pad, and nitrocellulose membrane are mounted together on an adhesive backing pad, which is often made of plastic.[89] Each pad overlaps with the next to ensure free fluid flow from one component to the next. Test and control zones are then prepared by loading the membrane with the necessary probes, thereby forming the conjugate pad—probes may include control, capture, or universal probes, depending on the type of lateral flow assay.[90,91] The final fabrication steps involve cutting the assembled, loaded device to size, and, for commercial production, encasement in a plastic housing for transport, durability, and handling. Furthermore, for digital-

readout lateral flow assays, electrochemical substances would also be added to the conjugate pad.[90]

B. Proposed fabrication methods for 2 D paper-based μPads

Paper-based microfluidic devices for diagnostic purposes can be manufactured in both two-dimensional (2 D) and three-dimensional (3 D) forms. 2 D paper-based microfluidic analytical devices operate by capillary action in the cellulose-based paper substrate.[92,93] When a hydrophilic sample is placed on the paper, it navigates through the hydrophilic paper structure between the hydrophobic lines and barriers formed through a patterning and deposition process. As a result, fluids can be transported and manipulated through horizontal and vertical patterns tailored to the complexity and requirements of the given analytical application.

Several approaches to the fabrication of paper-based microfluidic devices have been reported in recent studies, all of which utilizing the hydrophilic properties of the paper substrate and some form of hydrophobic ink deposited as a barrier. The fabrication methods can be broken down into analog and digital methods. Analog methods, which require the use of a prefabricated mask or plate, include (1) Photolithography, (2) Flexographic Printing, (3) Plasma Treatment, (4) Wax Patterning, (5) Screen Printing, and (6) Wet Etching. As opposed to analog methods, digital methods allow for near-instantaneous changes to the pattern; these methods include (7) Laser Patterning, (8) Inkjet Printing, and (9) Pen Plotting. For low-cost, point-of-care diagnostic devices, the ability to dynamically alter the pattern through digital means is a significant advantage.

For all of the following fabrication methods, the applicability to commercialization should also be considered. There are limits to the commercialization potential of paper-based microfluidic devices for urine assays. For instance, some methods can be costly and other methods or the chemicals involved would be difficult to scale up for commercial production. Another consideration is the stability of the finished device, which influences the mass producibility, storage, consistency, and accuracy of the device. These factors are largely dependent on the purpose of the device and reagents used in addition to environmental conditions such as humidity—even light can play a role when colorimetric detection methods are used.

1. Photolithography

Using photolithography, hydrophobic patterns are created on the paper substrate with an ultraviolet-polymerizable photoresist.[94] Using a photomask, this fabrication method forms patterns of micro-channels on the paper by restricting the polymerization of the photoresist to transparent areas of the mask [Fig. 7(a)]. This method can be used to produce low-cost paper-based microfluidic devices by using an inexpensive photoresist.[95] Another variation of photolithography was described by Martinez *et al*., who proposed a rapid prototyping of paper-based microfluidic devices via fast lithographic activation of sheets of chromatography paper that were soaked in SU-8 (photoresist) and baked.[96] This method relied solely on an ultraviolet lamp and a hot plate, which can even be substituted with sunlight, if necessary. This simple setup does not require the organic solvents or equipment typically used in photolithography.

2. Flexographic printing

This microfluidic device fabrication method is a direct variation of classic flexographic printing, which functions by rolling a flexible relief plate over the ink, then pressing the inked relief plate on a substrate. Polystrene is used as an ink for flexographic printing on paper substrates to form hydrophobic boundaries, which may partially or completely penetrate through the paper[97] [Fig. 7(b)]. The ability to manipulate the degree of penetration enables the design of very thin fluidic channels, which reduce the required sample volume for diagnostic devices. This fabrication method is also fully compatible with the roll-to-roll flexography printers used widely in printing houses, which makes it ideal for large-scale production.

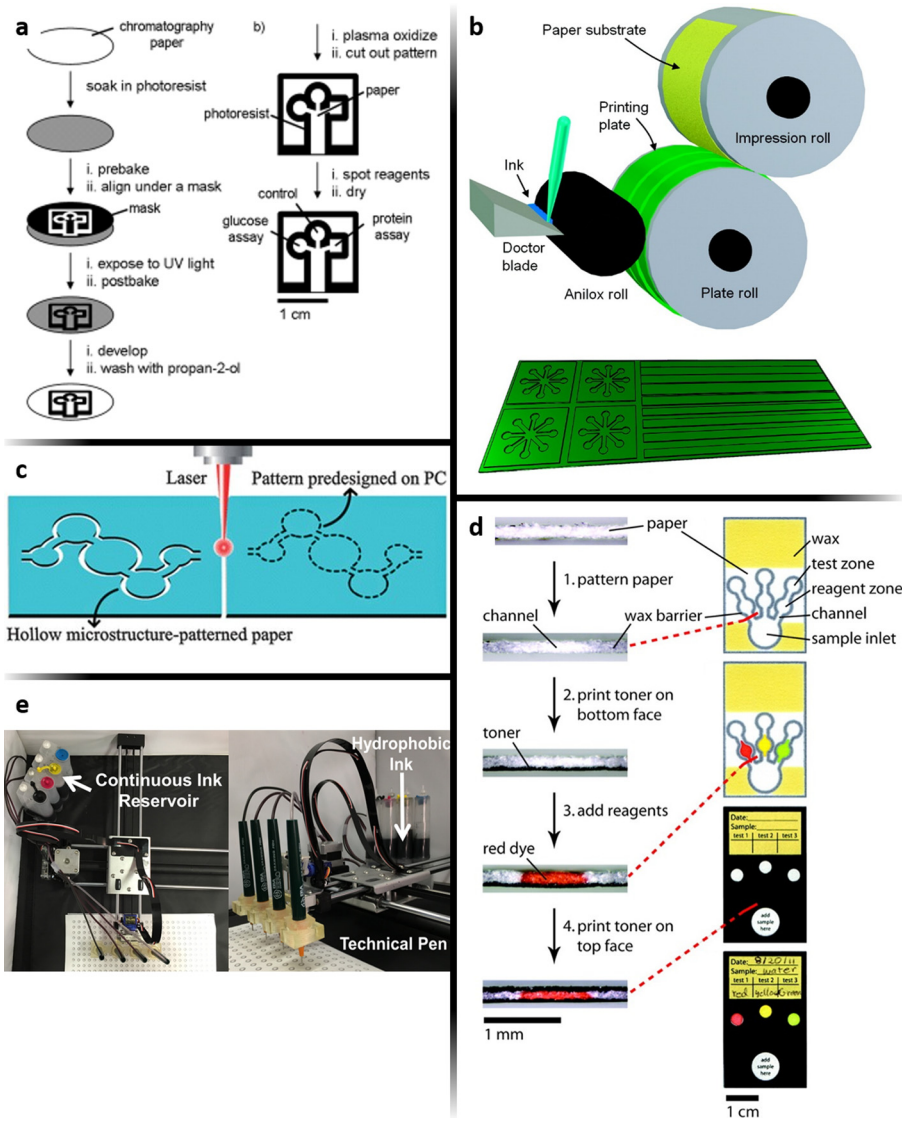

FIG. 7. Selected fabrication methods of two-dimensional paper-based microfluidic devices. (a) Schematic illustration of fabrication by photolithography. Photolithography method used to pattern photoresist embedded in a paper substrate, which is then modified for application to bioassays.[94] Reproduced with permission from Martinez *et al.*, Angew. Chem. Int. Ed. **46**(8), 1318–1320 (2007). Copyright 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (b) Schematic illustration of fabrication using a flexography unit with a printing plate containing the relief patterns that form the hydrophobic regions to be printed on the paper.[97] Reprinted with permission from Olkkonen *et al.*, Anal. Chem. **82**(24), 10246–10250 (2010). Copyright 2010 American Chemical Society. (c) Method of laser patterning, specifically paper cutting. A laser is used to cut through the paper to form channels.[111] Reproduced with permission from Nie *et al.*, Analyst **138**(2), 671–676 (2013). Copyright 2013 Royal Society of Chemistry. (d) Schematic illustration and cross-sectional images of the fabrication of fully enclosed paper-based microfluidic devices. First, hydrophobic barriers are printed by wax printing, then the reagent zones are filled with sample dye, and a toner layer is laser printed on both faces of the prepared device to seal and protect the device.[106] Reprinted with permission from Schilling *et al.*, Anal. Chem. **84**(3), 1579–1585 (2012). Copyright 2012 American Chemical Society. (e) A multi-pen plotter for fabricating paper-based microfluidics. Desktop pen plotter integrated with low-cost, 3 D-printed multi-pen holder, which was custom-designed to increase the throughput of the setup.[123] Reproduced with permission from Amin *et al.*, Anal. Chem. **89**, 6351 (2017). Copyright 2017 American Chemical Society.

3. Plasma treatment

Plasma treatment can be used to fabricate paper-based microfluidic devices. The process starts by making the paper hydrophobic through octadecyltrichlorosilane (OTS) silanization. The hydrophobic paper is then covered with a mask that has cutouts which reveal the channel

pattern to be treated with plasma. The hydrophobic molecules in the unmasked portions of the paper undergo degradation, thus uncovering the hydrophilic paper underneath.[98] After plasma treatment of OTS-silanized paper, the contact angle between water and the hydrophilic channel was measured to be 0°, representing full contact, while the contact angle of water on the hydrophobic region was 133.9° +/−1.3°.[98] Furthermore, the water not only made full contact with the hydrophilic channel but could also penetrate fully through the thickness of the paper within only 30 s.

The advantage of plasma treatment is that the patterning via masking enables simple design and fabrication of functional elements in the microfluidic device.[99] However, the drawback is that the plasma treatment often extends beyond the demarcated lines of the mask, although this can be remedied by careful control of the plasma intensity and exposure time.

4. Wax patterning

Wax patterning has certain virtues that make it ideal for prototyping or implementing low-cost, high-volume bioassays in remote or developing regions. These include facile printing and curing, quick production without advanced facilities, inexpensive materials, environmentally friendly process,[100–102] and compatibility with mass production of large batches.[103] Wax printing methods include painting on melted wax by hand using a pen, printing a pattern with a normal printer to be traced over with wax by hand, or direct printing of wax with a custom-designed wax printer.[104] The result is the formation of hydrophobic wax barriers on the paper substrate, which direct the flow of liquid through channels or into separate reaction zones. Lu *et al*. optimized the process such that it could be completed in under 10 min.[105]

In an advanced approach to the wax printing fabrication method, Schilling *et al*. demonstrated the capability of producing fully enclosed paper-based microfluidic devices.[106] After printing the hydrophobic barriers using traditional wax printing methods, a solid layer of toner was printed on both sides of the paper device using a laser printer. Unlike typical laser-printer ink, which partially saturates the paper, the toner used here remains on the surface, coating both the top and bottom surfaces of the device. The result is a fully enclosed microfluidic device in which the channels are protected from contamination, reagents deposited in the device are protected during storage, the effect of evaporation of the sample is reduced, and handling and operation are easier [Fig. 7(d)].

5. Screen printing

In general, screen printing is a process that involves squeegeeing an ink through a screen and onto a substrate directly beneath the screen. The screen itself is fabricated such that specifically designated regions are open, while others are sealed, producing a template through which the ink penetrates. Using a traditional screen-printing process, carbon-based electrodes can be printed directly onto cellulose paper to form electrochemical analytical devices.[107] By printing multiple designs on paper, Renault *et al*. demonstrated the use of a single pair of driving electrodes to control 18 separate screen-printed bipolar electrodes. Each of these bipolar electrodes reads out the electrochemical state, demonstrating the multiplexing capabilities of screen-printed electrochemical microfluidic devices.

Via screen printing, wax can be deposited as hydrophobic barriers, closely related to the wax patterning, as described above. Wax-based screen printing utilizes the typical screen-printing process, by which solid wax is deposited onto the paper surface through the screen pattern.[108] The wax is then melted using a hot plate so that it penetrates into the paper to form the final hydrophobic barriers. A significant limitation of wax-based screen printing is the resolution: the hydrophobic barriers produced by this method were 1200–1800 mm, while the hydrophilic channels were 550–1000 mm.[108] However, this fabrication method is very low-cost and environmentally friendly. Furthermore, there is no need for a clean room, ultraviolet light, complex, or expensive equipment, and does not make use of toxic or harmful solvents. Ideal for use in developing countries, this method only truly requires wax, screens, and a hot plate, or similar.

6. Wet etching

Wet etching of paper-based microfluidic devices is a two-step process.[109] Hydrophilic filter paper is patterned with a trimenthoxyoctadecylsilane solution to produce a hydrophobic pattern. The hydrophobic patterned paper is then covered with a mask containing NaOH, and then a 30% glycerol solution, which initiates chemical etching of the silanized filter paper. As a result, the unmasked regions become hydrophilic, while the portion covered by the mask remains hydrophobic; the product is hydrophobic barriers around the etched hydrophilic channels.

7. Laser patterning

Laser patterning for the fabrication of paper-based microfluidic devices involves the polymerization of a photopolymer by application of a laser [Fig. 7(c)]. This process results in narrow barriers and channels of 120 and 80 μm, respectively.[110] A carbon dioxide laser is ideal for this process, as it is able to both polymerize the photopolymer and cut the piece of paper in a single-step operation.[111,112] Three versions of laser patterning have been proposed: etching by removing hydrophobic materials, curing by polymerization, or direct cutting. Furthermore, attributed to the highly controllable nature of lasers, the process can be adapted for various surface elements and substrates, such as cellulose-based wicking pads or glass-fiber pads.[113] Laser etching can also prove to be a very inexpensive process.

8. Inkjet printing

Inkjet printing is a newer methodology for the fabrication of paper-based microfluidic devices, which takes both paper chemistry and the features of digital inkjet printing into account. Li *et al.* demonstrated the applicability of inkjet printing in the fabrication of bio-molecular patterns for microfluidic sensors.[114] In addition to traditional inkjet cartridges, custom cartridges can enable the precise delivery of biomolecules and chemicals into the micro-channels to form sensing regions within the printed patterns. The result is a fully functional sensing and analytical device. In general, inkjet printing fabrication techniques are quite scalable and adaptable for high-speed, high-volume, low-cost use with commercially available printing equipment.[114] Furthermore, this approach has been shown to provide quantitative, repeatable results.[95,115,116]

Xu *et al.* developed a micro-channel printer, which uses permanent marker ink as the hydrophobic barrier agent.[117] After the ink solvent evaporated, a hydrophobic resin remained on the paper substrate.

An alternative inkjet printing approach was reported by Abe *et al.* Rather than printing hydrophobic barriers onto hydrophilic paper, this method utilized a hydrophobic paper substrate and printed the hydrophilic channels directly.[118] Filter paper was coated with a 1% hydrophobic polystyrene solution, and on this substrate was printed a solvent as the "ink," which etched hydrophilic channels into the substrate. In a second pass, the printer deposited chemical reagents to make the final functional analytical chip.

The inkjet printing technique can also be implemented with conductive materials and electrodes to produce actively controllable microfluidic chips. Koo *et al.* created actuatable hydrophobic valves by inkjet printing and/or spraying a conductive layer of hydrophobic and hydrophilic electrodes.[119] By applying a potential to designated areas of the fabricated devices, most often on a channel barrier, the fluorinated monolayer on the electrodes can be polarized and thus made more hydrophilic, effectively opening the channel.

Rajendra *et al.* introduced a silicone-based inkjet printing platform that provides enhanced durability for biological assay applications.[120] The silicone-based ink is resistant to dissolution from aqueous surfactant solutions and can still be used in inexpensive thermal inkjet printers.

An omniphobic fluoro-alkylate paper was designed by Lessing *et al.*, which provides a superior substrate for high-resolution printing, enables printing of conductive patterns, and improves the durability.[121] This approach was used to fabricate paper-based microfluidic chips capable of being folded and exposed to common solvents, while still retaining their conductivity.

9. Pen plotting

Fabrication of paper-based microfluidic devices by pen plotting can be done with an electronic pen plotter system coupled with hydrophobic pens or markers. Our group leveraged the hydrophobic nature of permanent marker ink, specifically Comix broad-tip markers, to produce the hydrophobic barriers necessary for micropads.[122] The permanent marker was then attached to an AxiDraw desktop pen plotter to repeatably produce hydrophobic patterns on chromatography paper. This method is very low cost, as it only requires markers and a pen plotter system. Furthermore, pen plotting shows great potential for being a low-cost, high-throughput, and rapid-prototyping fabrication method.

Functioning on the same principle of using permanent markers to form hydrophobic barriers delineating hydrophilic zones, our group implemented a continuous ink system and a multiplexed pen holder [Fig. 7(e)].[123] A continuous-ink system was integrated to provide a continuous supply of ink to technical pens, thereby improving the cost-effectiveness and throughput of fabrication compared with the previous ink supply via fixed-volume, permanent markers. In the place of permanent marker ink, a commercially available hydrophobic solution was dyed and used as the plotting substance. Additionally, a custom-design multi-pen holder was 3 D printed to enable simultaneous plotting of several patterned devices in a single pass. Lastly, a lamination step was included in the fabrication process, in which the backsides of the paper devices were laminated to improve the stability, durability, and mechanical strength. The multiplexed, continuous-ink pen plotting approach was validated by performing colorimetric chemical and biological assays. As expected, the color increased as the analyte concentration increases for the each assay tested, demonstrating the applicability of this fabrication method to be used to create low-cost, high-throughput diagnostic devices.[123]

C. Proposed fabrication of 3 D paper-based μPads

Based on the same functionality principles underlying 2 D microfluidic devices, 3 D paper-based microfluidic devices have been developed, expanding the network of hydrophilic channels into a multi-dimensional format, but retains the pump-free, compact, low-cost advantages of paper-based devices. Multiple approaches have been proposed for the creation of 3 D paper-based devices.[124]

Martinez *et al*. demonstrated a layered approach, which is an advancement of the open-channel format of conventional paper-based microfluidics.[125] Lateral hydrophilic channels were demarcated by hydrophobic polymer barriers on paper sheets; vertical channels were added by layering these sheets with double-sided adhesive tape [Fig. 8(a)]. Each layer of the stacked microfluidic device can be comprised of different papers containing different chemical reagents, for instance, to combine multiple functions into a single device. Likewise, the holes cut into the tape layers can be specifically designed to control the vertical flow to the different regions of the paper layers. However, this paper-and-tape layered approach is susceptible to tape to weakening caused by aqueous solutions of dyes, chemicals, and samples.

He *et al*. demonstrated an alternative approach by which a stereolithographic 3 D mask was used in a photo-curing process to rapidly produce paper-based microfluidics.[126] This process demonstrated the ability to fabricate 3 D microfluidic devices in only 2 minutes, due to the fact that all hydrophobic barriers of each layer were cured at the same time.

Another alternative approach to 3 D micropads, reported by Liu and Crooks, utilized the principle of origami paper folding to fabricate 3 D devices.[127] The entire device started as a single, flat sheet and a photolithographic process was used to pattern the hydrophilic channels for each layer in a single step [Figs. 8(b) and 8(c)]. After patterning, the paper was folded to form the 3 D paper-based microfluidic device. Ge *et al.* implemented this origami-based fabrication method for an immunodevice to perform blood plasma separation, automated rinsing, and multiplexed detection.[128]

VIII. APPLICATION TO URINE ANALYSIS

Paper-based microfluidic analytical devices are of great use for urine analysis. A valuable tool in healthcare is the clinical analysis of urine due to its efficiency, affordability, and

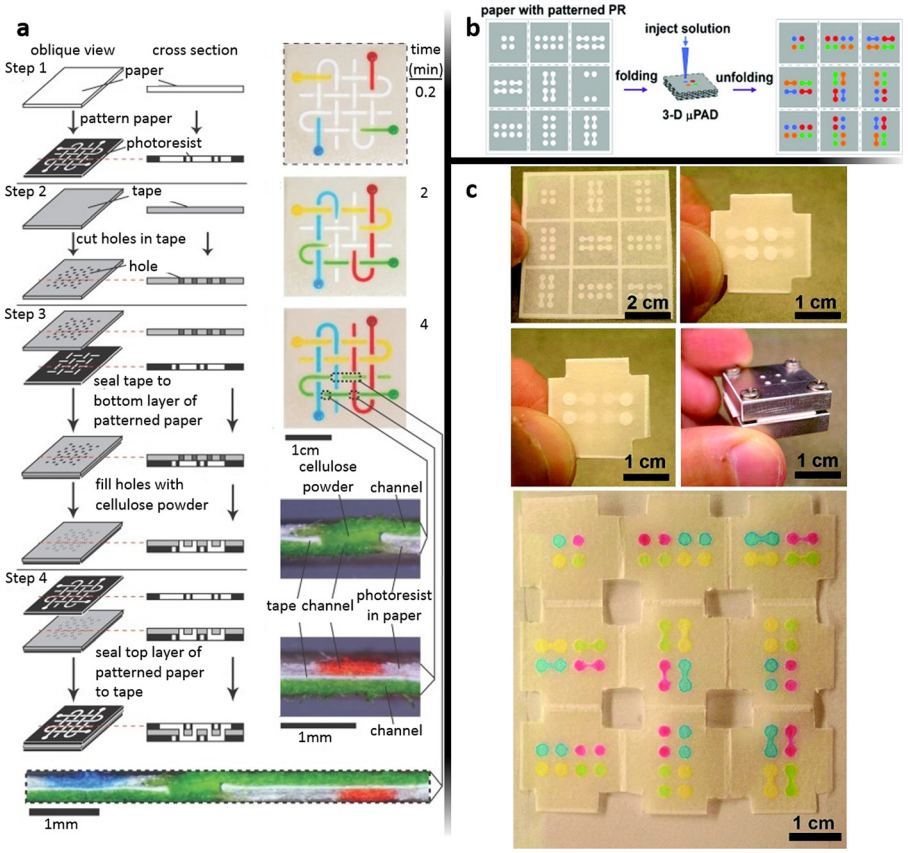

FIG. 8. Three-dimensional paper-based microfluidic devices. (a) Schematic illustration of the fabrications steps of a 3 D paper-based microfluidic device constructed from the layering of photolithography-patterned paper and cut tape.[125] Top-view and cross-sectional images show the three-dimensional flow of samples through the device, in both the horizontal and vertical direction. Reproduced with permission from Martinez *et al*., Proc. Natl. Acad. Sci. **105**(50), 19606–19611 (2008). Copyright 2008 The National Academy of Sciences of the USA. (b) Schematic illustration of the conceptual origami paper-folding based 3 D microfluidic device.[127] (c) Images depicting the device patterned as a single flat piece of paper, the device in its folded state, a holder for the device, and the resulting distribution of samples once the device is unfolded.[127] Reprinted with permission from H. Liu and R. M. Crooks, J. Am. Chem. Soc. **133**(44), 17564–17566 (2011). Copyright 2011 American Chemical Society.

non-invasive methodology.[129,130] Urine analysis is useful for diagnosing disease, detecting organ failure or malfunction, testing for drug abuse and exposure to toxins, etc. There are three main approaches to the standard urinalysis panel: observation of physical characteristics, microscopic investigation, and biochemical analysis. While microfluidic devices prove to be beneficial for some biochemical assays, paper-based devices offer the same critical functions with the added benefit of being low-cost, easy to manufacture, and disposable. Notable urine assays in this category are discussed herein: chemical assays are traditionally performed by test strips, lateral flow assays, drug testing, and disease-specific protein biomarker tests. Each of these urine-based assays has the potential to be redesigned, redeveloped, and implemented in the form of paper-based microfluidic devices. For the purpose of this review, the tests are organized by the formats in which they are presently available.

A. Traditionally test strip-based chemical assays

Common chemical assays may be carried out by test strips, but it is important to note that paper-based microfluidic devices show commensurate capability and performance in these applications. While these chemical assays are most typically performed via test strips, paper-based microfluidic devices show great promise to fulfill the same role as test strips for these assays. A selection of these assays is presented as follows:

1. Commercially available test strip assays

a. Creatinine assay. Derived from creatine phosphoric acid, creatine non-enzymatically dehydrates to form creatinine as a metabolic waste product, which then leaves the body through the urinary system.[131] Kidney dysfunction may be indicated by the renal glomerular filtration rate, which can be determined by measuring the creatinine levels in urine. Correlating severe kidney disease, moderate dysfunction, and mild organ failure to creatinine clearance levels of 20%–40%, 40%–60%, and 60%–80%, respectively, can be helpful indicators of renal function.[132]

There are also several applications in which creatinine levels can be used to help produce more useful results for other assays. Specifically, when testing for pesticides, metals, drugs, or metabolites, their levels in the urine are normalized to the creatinine concentration to compensate for variable factors such as dilution and water content.[133,134] Similarly, the albumin-to-creatinine ratio can be used as a correction factor to corroborate the results of albumin assays and confirm the presence of microalbuminuria, as the creatinine level is related to the size of the patient[135–138] (See Protein Assay, below). Furthermore, the lactate-to-creatinine ratio can be an early indicator of an infant's risk for hypoxic-ischemic encephalopathy.[139]

b. Glucose assay. Glucose monitoring is of crucial importance for people suffering from diabetes. As the primary energy source for humans, glucose is generated by the body through the digestion of carbohydrates. For people with diabetes, their body's inability to properly manage glucose necessitates regular monitoring and, at times, medical intervention to prevent severe health complications. Micro-analytical tools allow patients with diabetes to monitor glucose regularly, reducing the possibility of developing diabetic ketoacidosis (DKA), hypoglycemia, and diabetic coma.[140]

c. Ion detection. Inorganic ions—namely, sodium, potassium, calcium, magnesium, chloride, and phosphate—are commonly found in human serum and urine. Abnormal concentrations of these ions can be key indicators in the diagnosis of diabetes, liver dysfunction, kidney dysfunction, and cardiac disease, among other disorders.[141] Specifically, abnormally high calcium levels may indicate endocrine disorders, cancer, or osteoporosis. Conversely, low calcium levels may be indicative of a vitamin D deficiency, hypoparathyroidism, or diseases relating to poor absorption of nutrients.[142]

d. Ketone assay. Patients who suffer from type-1 diabetes often develop DKA as a result of the condition, which is associated with higher-than-normal levels of ketones in the blood and urine due to low insulin levels. Ketones may also be present in urine in cases of malnutrition, including starvation and low-carbohydrate intake. Ketones levels appear elevated in urine samples before they do in blood samples, so diagnosis can be accomplished more proactively via urine analysis.[140]

e. Lactate detection. Lactate detection is another example of tests, which are more efficient in urine samples than in blood. Urinary lactate concentration changes dramatically when the blood lactate concentration deviates from the normal range, while the blood lactate concentration itself is constantly varying.[143] This phenomenon can be exploited for lactate monitoring in people suffering from type-I glycogen storage disease. For these patients, treatment includes maintaining a continuous supply of glucose as well as lactate monitoring to monitor the success of the dietary treatments used.[143–148] Another application of urinary lactate detection is early diagnosis of newborns at risk for hypoxic-ischemic encephalopathy, by using the urinary lactate-to-creatinine ratio as an indicator.[139]

f. pH measurement. Urine may be classified as acid or alkaline based on its pH, which is of concern when it lies outside the healthy urine pH range between 4.6 and 8.0, with the average being 6.0. Urine may become overly acidic due to ketosis, diarrhea, metabolic acidosis,

respiratory acidosis, or large quantities of meat and fruit consumption. Conversely, alkaline urine may develop due to a primarily vegetarian diet, urinary tract infection, metabolic alkalosis, and respiratory alkalosis.[149–152]

g. Protein assay. The kidney maintains the levels of beneficial proteins in the blood, such as albumin, while also functioning to filter out waste products from the blood. When the kidney is seriously injured, excess amounts of albumin, which is a major protein component in plasma, may be filtered out of the blood.[153,154] By quantifying the albumin excreted from the body through urination, critical information about kidney performance can be deduced, which can often be linked to certain diseases.[155,156] As an example, an increased albumin excretion rate, known as microalbuminuria (MAU), can be an indicator of cardiovascular disease.[157,158] In patients with diabetes or hypertension, MAU can also be an early signal for nephropathy, which, when diagnosed at an early stage, may be reversible by careful monitoring and control.[154,159,160] In addition to albumin, abnormal concentrations of other urinary proteins can be identified to differentiate between various renal diseases such as MAU, nephrotic syndrome, subnephrotic range proteinuria, tubular proteinuria, and clinical albuminuria.[161,162]

h. Uric acid detection. When purine is metabolized by the body, uric acid is formed as a metabolic product and is excreted as a large component of human urine. However, excessive amounts of uric acid in urine is often linked to urinary stones or gout, among other disorders. Testing for uric acid is a significant diagnostic tool used for patients with purine metabolism disorders.[163]

2. Developing assays based on reagent spotting

These chemical assays, which have traditionally been performed by dipsticks and test strips, have recently been applied to paper-based microfluidic devices. The colorimetric assays previously found in the dipstick format have been adapted to paper-based microfluidic chips, which have further been applied to urine analysis. For example, Klasner *et al.* used photolithography to fabricate paper-based devices that applied the same colorimetric reagents for ketones and nitrite as dipsticks to detection pads in the device, which acted in a similar manner to the test pads of a dipstick.[140]

Many other researchers have pursued applications of paper-based microfluidic devices to urine analysis based on the foundations of reagent spotting on test strips. Similar to how test strips comprise several individual test pads, each designed to identify a different analyte of interest, paper-based microfluidic devices have also been designed for multiplexed bioassays.[94] In this approach, a microliter of urine is applied to the device and migrates through the paper channels into discrete test zones containing spotted reagents. In an effort to improve the colorimetric results, Dungchai *et al.* combined multiple indicators for a single analyte into a paper-based device, thereby providing greater resolution in the detection of various analyte concentrations.[51] To quantify urine analysis results, Martinez *et al.* fabricated paper-based microfluidic devices and functionalized the devices with reagents for colorimetric assays; the assays were then imaged using a smartphone and the captured images were analyzed to correlate the detected color to the concentration of glucose and protein in artificial urine.[161] Reagent spotting has also been implemented in 3 D paper devices, which utilize one of the many fabrication methods and borrow from the basic principles of origami. Sechi *et al.* reported a 3 D paper-based device, which was tested clinically and for measuring glucose and protein in urine samples.[164]

B. Lateral flow assay for urine analysis

1. Currently available lateral flow assays

Lateral flow assays offer the useful ability to test specifically for an analyte of interest using antibodies and lend themselves to unique urine analysis applications. Most notable and common are the so-called "pee-sticks," which test for pregnancy, ovulation, and menopause.

However, there is an abundance of assays that may be performed in the lateral flow assay format: bacterial and viral infections, metabolic disorders, toxic compounds, and other miscellaneous biomarkers.[165] Disorders such as diabetes, cardiovascular diseases, renal disease, prostate issues, and coronary syndromes can be detected, some with specificity high enough to provide detailed tracking of the disorder's progression. Antibiotics, pesticides, and various chemical residues can also be detected. Other applications of lateral flow assays include checking for previous infection or vaccination based on antibodies present, detection of pregnancy hormones, ovulation hormones, and menopause hormonal changes. While this list is not extensive, it demonstrates the current scope and future potential for applications of lateral flow assays for urine analysis.

2. Developing lateral flow assays

In addition to the aforementioned common applications of lateral flow assays, several other assays have been reported.[165] Lateral flow assays have been designed for the detection of infectious agents; some assays have been reported with extremely high sensitivity, only requiring as few as two cells for the detection of *Bacillus anthracis*.[165] Many metabolic disorders have been shown to be diagnosable using lateral flow assays: albumin is used as a biomarker for diabetes, and human serum in urine has been used as an indicator for renal disease.[165] Additionally, lateral flow assays have been implemented for the detection of toxic compounds and other miscellaneous applications. Developments have also been made in the physical structure of lateral flow assays despite their well-established format. For instance, Fenton *et al*. developed a multiplexed lateral flow assay based on various shapes and designs including a starburst and a branched tree.[166] These multiplexed lateral flow assays can be used to detect multiple analytes simultaneously from a single sample.

C. Drug testing

Drug testing by micro-analytical devices provides low-cost, reliable, and rapid results, with varied applications ranging from clinical diagnostics to forensic investigation to pharmaceuticals.[167] Of great concern, drug abuse and the over-prescription of drugs—particularly narcotics and stimulants, such as amphetamines, methamphetamines, codeine, and morphine—may have serious physiological effects on the drug user. Such inadvertent effects may include excessive nervous system stimulus, hindered breathing due to relaxed bronchial muscle, and the secretion of gastric acid, in addition to several other toxic and dangerous symptoms.[167–172] On top of these physical and biological side effects, social issues may develop due to the drug abuse and overuse, increasing the demand for facile drug testing. This need may be addressed using paper-based devices in the coming years.

IX. BIOMARKERS FOR URINE DIAGNOSTICS

A. Disease-specific protein biomarkers

The urinary proteome is rich in biomarkers for specific diseases (Fig. 9).[173] Proteins found in urine include those secreted by the kidney and urinary tract, but mainly include proteins that have been filtered from plasma. Since urine collection is noninvasive and can be performed continually and routinely, it is an ideal medium to detect and track biomarkers. Furthermore, urinary biomarker analysis is not only easier than that with blood but, due to the composition of the urinary proteome, researchers and doctors can gain insight into both the urinary system and the body as a whole.

In light of expanding proteomic techniques and rising interest in the urinary proteome, there are now over 1500 proteins that have been identified in normal human urine.[174] However, not all of these proteins are useful in biomarker analysis. First, in order to be useful for disease detection, the biomarker must be disease-specific, and not just a common sign or symptom. Second, the biomarker must also be consistent among individuals in order to provide accurate results. Following these guidelines, Shao *et al*. compiled the Urinary Protein Biomarker database, compiling results from many urinary protein biomarker studies in a readily available

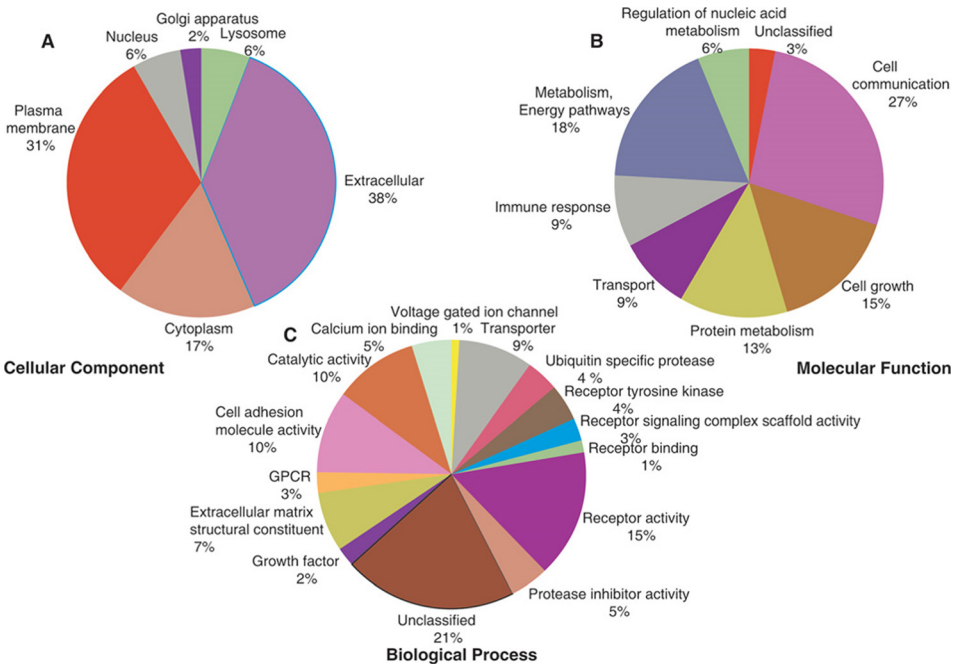

FIG. 9. Classification of proteins found in the urinary proteome classified based on the (a) cellular component, (b) molecular function, and (c) biological process.[186] Reprinted with permission from Marimuthu *et al.*, J. Proteome Res. **10**(6), 2734–2743 (2011). Copyright 2011 American Chemical Society.

format.[173] The Urinary Protein Biomarker database stores urinary biomarker discoveries published in PubMed, including peptide sequences and protein names, which allows for further research into the functions of the identified proteins. The database contains biomarker information for 10 cancers, 31 urological diseases, and 15 non-urological diseases, all found in the human urinary proteome. Disease-specific biomarkers were identified for bladder cancer, prostate cancer, pancreatitis, diabetic nephropathy, and necrotizing enterocolitis. In addition, there are also other proteins which are reliably related to multiple diseases. These multiple-disease-specific proteins include albumin, β-2-microglobulin, Zinc-α-2-glycoprotein, neutrophil gelatinase-associated lipocalin (NGAL), and α-1-microglobulin/bikunin precursor (AMBP).[173] Knowledge of these disease-specific protein biomarkers coupled with the capabilities of paper-based microfluidic devices has great potential for next-generation point-of-care or in-home diagnostic devices.

B. Emerging progress in biomarker research

However, current state-of-the-art disease diagnosis is limited by our knowledge of these diseases. A major component of efficient and effective patient care, beyond developing diagnostic tools, is identifying specific biomarkers for human disease which can be tested for. Recent urinary biomarker research is offering great insight into diagnosing and treating human diseases, disorders, and even cancers.[175,176] Many of the proteins commonly used for assays in blood plasma are also found in urine.[175]

While protein remains one of the most commonly used biomarkers for diagnostics and pathology, other biomarkers are emerging as promising alternatives. For instance, microRNAs are short chains of non-coding RNA that can be found in various biological fluids including urine. MicroRNAs are prevalent in extracellular vesicles, known as exosomes, and can be used as a biomarker due to their tendency to fluctuate in patients inflicted with some diseases.[175] In a novel method demonstrated by Ymir Genomics, intact exosomes from human or animal urine can be isolated, thereby procuring high-quality proteins and RNAs for use in biomarker analysis.[175] Exosomes are isolated from urine by one of the two methods: a proprietary molecule that causes the exosomes to precipitate, or by a capture resin which is already available.[177]

There are several advantages to this urine-based RNA biomarker method, including the samples' high stability for prolonged periods of time, the low-cost and rapid nature of sample collection and analysis, and the ability to collect high-quality proteins and nucleic acids from dilute urine samples. Current practices focused on microRNAs are blood-based, which inherently involves a more invasive, uncomfortable sample collection procedure than urine-based testing. Vesicles in a patient's blood are collected, and the RNA trapped inside is extracted and analyzed. For instance, RNA found in these vesicles has been used to diagnose non-small cell lung cancer by detecting gene mutations.[177] As opposed to commonly used immunohistochemistry and immunofluorescence, analyzing the RNA from exosomes requires no invasive biopsies. Ranging 30–100 nm in diameter, exosomes are found in most bodily fluids, but only recently has urine presented itself as a prime candidate for exosome collection and analysis.[177] As a result, microRNA testing may have the prospect of becoming more compatible with point-of-care testing, as urine is easily collected. With further research, paper-based tests may emerge to close the final gap between microRNA testing and accessible health diagnostics.

X. CONCLUSIONS AND FUTURE PERSPECTIVES

Preventive, evidence-based, person-centered care is the imminent future of medicine. Shifting away from the reactive approaches of today will require extensive research and development of affordable and accessible transformative technologies to perform regular—daily, hourly, or even continuous—health data measurements in the general population. Scientific innovation can bring about the sought after next-generation technologies capable of improving the well-being of patients and making healthcare more accessible.

Paper-based microfluidics are a promising technological innovation that will likely contribute to this paradigm shift. Microfluidic devices have already been proven as useful analytical tools and are significant contributors in preventative care. Unlike glass- and polymer-devices, paper-based microfluidic devices boast low-cost implementation, facile fabrication, and disposability while offering the same diagnostic capabilities as their traditional counterparts. Furthermore, there is a great diversity of fabrication methods available, some of which are ideal for commercial mass-production. In particular, digital fabrication methods enabling on-the-spot design, manufacturing, and implementation of paper-based microfluidic devices will facilitate rapid innovation in this field.

Drawing on the benefits and capabilities of paper-based microfluidics, urine is a promising medium for regular health analysis given both its extensive biometric applications and its ready availability and practicality for routine testing. Due to the nature of urine, it can be sampled in large volume and without the need for needles or special equipment. Urine can also be continually sampled easily over a longer period of time for continued monitoring of biomarkers. Furthermore, handling and disposal is simpler with urine than with since it is not considered infectious or biohazardous. Expanding upon the common urinalysis tests, ongoing research is demonstrating the use of protein and microRNA biomarkers for diagnostics.

With continued research, easily accessible, disposable, and highly effective paper-based microfluidics can be developed for urine analysis. Such devices may eventually be able to replace the current commercial rapid tests. Paper-based microfluidics represents a promising new direction: they have already been shown to possess the precise diagnostic capabilities that current test strips and lateral flow assays lack. With only the price differential remaining as a significant barrier to the commercial production of microfluidic devices, further research into fabrication techniques and the use of paper substrates promises to aid in the development of inexpensive microfluidic devices for diagnostic applications.

ACKNOWLEDGMENTS

S.T. acknowledges the American Heart Association Scientist Development Grant (No. 15SDG25080056) and the University of Connecticut Research Excellence Program award for financial support of this research. S.K. acknowledges the support of the National Science Foundation Graduate Research Fellowship (No. DGE-1247393).

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